EXPERIMENTAL INOCULATION OF BALB C MICE WITH EBOLA VIRUS

By

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(Under the direction of Dr. Donald Dawe)

ABSTRACT

Ebola virus is the causative agent of a severe hemorrhagic fever with fatality rates in humans approaching 90% with the Zaire species of the virus. Previous attempts at producing an experimental mouse model for Ebola Zaire have had limited success. Until recently, Ebola Zaire has not been shown to be lethal in adult BALB/c mice but has been lethal in suckling BALB/c mice. We performed blind passages in suckling, aged suckling and adult BALB/c mice with a low passage strain of Ebola Zaire. Suckling BALB/c and aged suckling BALB/c mice were susceptible to Ebola infection while adult BALB/c mice showed no susceptibility to Ebola infection. Antigen detection by ELISA indicated virus replication throughout the blind passages in all mouse groups. Plaque titrations in Vero E6 cells of passaged tissue suspensions were limited in success with only 8 of 43 suspesions producing plaques. Antigen detection by ELISA titers showed no correlation with the ability of the passaged suspension to produce viable plaques. The reason for the inability to produce viable plaques is unknown at this time. A more thorough evaluation of Ebola Zaire virus replication in mice is needed to fully understand Ebola Zaire virus replication in BALB/c mice.

INDEX WORDS: Masters Thesis, Ebola, hemorrhagic fever, BALB/C mice, mouse model

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DEDICATION

I would like to dedicate this thesis to my loving mother, Charlotte Stockton. Without her love, encouragement and support, through out both my undergraduate and graduate studies, this would not be possible. Her love, encouragement and support were and still are the greatest gift a son could ask for. Thank you mother.

I would also like to dedicate this to my wife, Kimberly, hoping that the results of completing this thesis will assist in our attempt to achieve a lifetime of happiness.

ACKNOWLEGMENTS

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Subtypes and Morphology

Ebola (EBO) virus is the causative agent of a severe hemorrhagic fever with fatality rates approaching 90% with the Zaire species of the virus. Ebola virus is an RNA virus and a member of the genus filoviruses and family Filoviridae. Filoviruses are very similar in morphology and are separated into two genera, Ebola and Marburg. The Ebola genus consists of Zaire (EBO-Z), Sudan (EBO-S), Reston (EBO-R) and Ivory Coast (EBO-IC) (Kiley et al. 1982; Feldman et al. 1992; Murphy et al. 1978). Marburg subtypes consist of a single species of Marburg Virus. Although originally suspected to be a rhabdoviruses, filoviruses are sufficiently distinct from the other non-segmented negative-stranded RNA viruses to warrant taxonomic status as a separate virus family within the order of Mononegavirales (Murphy et al. 1978). Their basic structure is long and filamentous. The particles are pleomorphic, meaning they can exist in many shapes but may be curved (U) or hooked (6) shaped.

History of Ebola

The earliest records of a filovirus were documented as laboratory infections in Marburg, Germany and Belgrade, Yugoslavia (Kissling et al. 1968; Martini et al. 1971). Laboratory personnel had been working with African green monkeys, exported from Uganda, and their tissues and became infected. Over thirty individuals became ill with hemorrhagic fever and seven died. The earliest recorded outbreaks of Ebola virus were reported in 1976 during two separate outbreaks in Zaire and Sudan (World Health

Organization, 1978; World Health Organization, 1978; Johnson et al. 1977). These were significant outbreaks resulting in over 550 confirmed cases and over 300 deaths. More recent outbreaks, Gabon 1994 (44 cases, 28 deaths, originally diagnosed as a yellow fever outbreak), Zaire 1995 (315 confirmed cases, 244 deaths), Gabon 1996 (60 confirmed cases, 45 deaths), South Africa (2 confirmed cases, 1 death), Uganda 2000-2001 (425 presumptive case-patients, 224 deaths) and again Gabon 2001-2002 (60 confirmed cases, 45 deaths and still on going) have brought the devastating disease to the forefront (Amblard et al. 1997; Sanchez et al. 1995; World Health Organization, 1996; World Health Organization, 1996; World Health Organization, 1997; World Health Organization, 2001).

Existing Ebola Animal Models

Since the discovery of the virus there were two existing models to study Ebola hemorrhagic fever, a monkey model and a guinea pig model (Ellis et al. 1978; Bowen et al. 1978; Bowen et al. 1980). Both have distinct advantages and disadvantages as compared to each other and other possible models. The primate model holds the advantage of an infection that mimics the human disease but has the disadvantage of the expense to house and maintain large numbers of monkeys. This economic disadvantage is becoming more relevant today with the ever increasing transport and safety regulations required for wild-caught monkeys and the high cost of facility bred monkeys (Centers for Disease Control, 1990). The disadvantage of the guinea pig model is that it does not mimic human disease and there are less reagents to work with when compared to monkey and other potential models. On the other hand, guinea pig models have shown that after initially developing a non-lethal illness that subsequent passing of the virus in guinea pigs

increases the virulence until a uniform lethal dose is attained (Bowen et al. 1980; Connelly et al. 1999).

Purpose of the Study

Previous attempts in adapting Ebola strains have been limited because of the stringent biosafety requirements needed to safely handle the live virus and infected animals (Centers for Disease Control/National Institutes of Health). Some of these limited experiments have shown that the EBO-Z strain of virus can cause a lethal infection in newly born suckling BALB/c mice (McCormick et al. 1983). Adult BALB/c have been shown not to be susceptible to EBO infections (Swanepoel et al. 1996). However, a recent publications indicates that it is possible to adapt EBO-Z in adult BALB/c mice (Bray et al. 1998; Gibb et al. 2001). We have therefore have undertaken this study to try to duplicate this method with the CDC stock of EBO-Z.

CHAPTER 2

MATERIALS AND METHODS

Facility and Personnel

All studies were carried out in a Maximum Containment Laboratory (MCL) at the Centers for Disease Control and Prevention, Atlanta, GA. This MCL is a Biosafety Level 4 (BSL 4) laboratory as described in the U.S. Department of Health and Human Services: Biosafety in Microbiological and Biomedical Laboratories, 4th Edition (Centers for Disease Control/National Institutes of Health). All personnel involved had extensive training in the appropriate techniques for handling and working with animals and BSL 4 agents.

Virus

Ebola virus, strain Zaire, was isolated from a woman in 1976 and then passed once in Vero E6 cells. The strain of Ebola Zaire 1976 had no previous passage history in mice and is thus considered a low passage (LP EboZ 76) strain. The virus suspension has a titer of 4×10^6 pfu/ml and the stock cultures were maintained in liquid nitrogen tanks.

Mice

BALB/c mice, aged from 1 day to 8 weeks were purchased from a commercial source (Harlan Sprague Dawley, Inc., Indianapolis,IN). Mice were kept in a Maxi-Miser caging system utilizing HEPA filters (Thoren Caging Systems Inc., Hazelton, PA). Mice were allowed access to food and water ad libitum.

Passage in Mice.

Mouse lineages of different ages were tested: adult, suckling and aged suckling BALB/c mice. Adult BALB/c mice (AB) were 8-12 weeks old, aged suckling mice (ASB) were 9-12 days old and new born suckling mice (SB) were 1-3 days old at the time of inoculation. Each set inoculated included five to ten suckling mice or aged suckling mice/litter or five adult mice per passage. All inoculations were intraperitoneal (IP), 0.5 ml for adults and 0.05 ml for suckling or aged suckling mice. Mice were monitored for signs of disease on a daily basis over a two-week period. Weight for each adult mouse was also monitored and tabulated daily over a two-week period. Moribound animals from each group were anesthetized then euthanized and spleens and livers were removed to create suspensions for testing and further passage. Surviving mice were anesthetized then sacrificed and spleens and livers harvested. All spleens and livers were removed aseptically, weighed and then ground in sterile sand and resuspended in MEM (Gibco) at 1:10 weight/volume aliquoted and held at –70 C for future determination of their state of infection and/or subsequent passages. Each subsequent passages of virus involved IP injection of virus from an organ suspension stock, described above, derived from a single adult mouse or a pooled stock suspension of suckling or aged suckling mouse tissues.

ELISA

All suspensions were tested for antigen content by an antigen capture ELISA technique (Ksiazek TG. 1991; Ksiazek et al. 1992). All aliquots of suspensions handled outside of the MCL were also subjected to 5 x 10^6 rads (50,000 gy) gamma-irradiation in dry ice for safe handling outside of the MCL. Ninety-six well microtiter plates were

equally divided into positive (upper) and negative (lower) halves. Positive wells were coated with a 100µl mixture of 7 monoclonal antibodies, anti-Ebola MAb 7-mix (EBO-Z and EBO-S VP40 specific, EBO-Z GP specific and EBO-S NP specific) while negative wells were coated with 100µl of ascitic fluid from mice injected IP with myeoloma cells. The monoclonal antibody mix and ascitic fluid were both diluted 1:1000 (optimal dilution determined by checker board titration, Appendices A) in coating buffer (PBS pH7.4 with 0.001% thimerosal) and coated plates were held at 4 C for at least 16 hours to allow for sufficient binding to the plate. Plates were first washed with a Skatron ELISA plate washer utilizing three 250ul wash/rinse cycles with a PBS, ph 7.4, 0.1% Tween 20 washing solution. After washing, four fold serial dilutions (1:4-1:256), final volume of 100µl, of passage material in PBS/skim milk were then placed in each half of the microtiter plates and incubated for 60 minutes at 37 C. Plates were then washed as described above and 100µl of a rabbit anti-EBO-Zaire, Sudan and Reston serum cocktail diluted 1:1500 (determined by cross titration, Appendices B) in PBS/skim milk was added to all the wells. Plates were again incubated for 60 minutes at 37C. Plates were then washed as described above, and 100µl of an HRP (horse radish peroxidase) labeled goat anti-rabbit conjugate (BioRad, Goat Anti-Rabbit IgG (H&L) catalog number 170-6491) diluted in PBS/skim milk was added at a dilution of 1:10,000 to all wells and incubated for 60 minutes at 37C. After incubation and subsequent washing, 100µl of equal parts of ABTS Peroxidase Substrate and Peroxidase Solution B (Kirkegaard and Perry, catalog number 50-62-01) were added and incubated for thirty minutes at 37 C and then immediately read. Plates were read using dual wavelengths, consisting of 410 and 490 nm, using a Dynatech MR5000 ELISA plate reader. Corresponding wells from the

positive and negative halves of the plate were subtracted from each other to arrive at four different individual dilution OD's (optical density) and the four dilutions were then summed to reach a final OD. An individual dilution with an OD greater than 0.10 nm was assigned a titer corresponding to that dilution. A titer of $\geq$ 1:16 with an OD sum of > 0.45 nm was considered positive.

Virus Titration

Vero E6 cell monolayers grown in roller bottles using Eagle's Minimal Essential Media, (MEM, Life Technologies/GIBCO) with 10% heat inactivated fetal bovine serum (FBS, Hyclone) and 1% Penicillin/Streptomycin were rinsed with phosphate-buffered solution (PBS, GIBCO) pH 7.4 to remove excess FBS. Vero cells were then trypsinized using Trypsin-EDTA (Life Technologies/GIBCO) for less than 10 minutes and an equal volume of FBS was then added to neutralize the Trypsin-EDTA. Cells were then resuspended at 5 x 10^5 /ml in MEM with 10% FBS and 1% Pen/Strep. Two tenths of an ml of the cell suspension was placed in each well of a Costar 48 well cluster plate and incubated over night at 37C to allow for confluency. On day two, medium was aspirated off using a multi-channel pipette and in duplicate, 0.1 ml of ten fold serial dilutions (10^{-1} through 10^{-8}) of harvested tissue suspensions made in EMEM (Life Technologies/GIBCO) with 2% FBS and antibiotics was allowed to adsorb onto the monolayer for 45 minutes in a humidified CO2 chamber at 37C. Equal volumes of 2x Basal Modified Eagle's Medium (BMEM; Life Technologies/GIBCO) and 2% methyl cellulose (MC, Sigma) suspension were combined for a final 1x BME/1% MC. Six tenths of an ml of the 1x BME/1% MC was added to each well. Plates were then placed back in a humidified CO2 chamber at 37C and were incubated and observed for 7 days.

On the seventh day, medium was aspirated off using a multi-channel pipette and 1.0 ml of fixing solution, 95% ethyl alcohol and 5% glacial acetic acid, was added and allowed to stand at room temperature for 10 minutes. Fixing solution was then dumped from the plates and the fixing procedure repeated one time. Plates were allowed to air dry and were then packaged in a double heat sealed bag. Heat sealed bags were removed from the MCL through a Lysol dunk tank, then placed on wet ice and were subjected to 5×10^6 rads (50,000 gy) gamma-irradiation for safe handling outside of the MCL. Fixed and irradiated plates then were held at 4C until immunostaining.

Immunostaining

Indirect immunostaining for Ebola virus was used for detecting antigen. Plates were rinsed, using slow flow tap water, 6-7 times and tap dried. One tenth of an ml of anti-EBO polyclonal rabbit serum, diluted 1:1000 in PBS/skim milk was added to each well. Plates were incubated at room temperature with rocking for 30 minutes. Plates were again rinsed and tap dried. To detect the bound anti-EBO antibodies, 0.1ml, 1:2000 suspension, of a commercial grade HRP labeled goat anti-rabbit IgG conjugate (BioRad, Goat Anti-Rabbit IgG (H&L)) was added to each well. Plates were again incubated and rocked for 30 minutes at room temperature. Plates were then rinsed and tap dried. One tenth of an ml of True Blue (Kirkegaard & Perry) peroxidase substrate was added and plates were incubated at room temp for 25-30 minutes. Plates were then rinsed 3-4 times and allowed to air dry. Once plates were completely dry, blue stained plaques were counted.

CHAPTER 3

EXPERIMENTAL RESULTS

Experimental Mouse Passage Lineage

Mouse passage lineage and results can be seen in Figure 1. Tissues for each SB experimental passage were passed in another group of SB as well as ASB and/or AB groups. SB and ASB did show signs of illness and some death occurred. The AB groups did not show any signs of illness and no deaths occurred.

SB Passages

Suckling mice did show susceptibility to Ebola infection. By day eight post inoculation (PI) of initial passage in SB, one suckling mouse had died due to illness and six others were showing signs of illness. Tissues of the six sick and one dead mouse were harvested and pooled for next passage. During the next two passages (SB2-SB3), suckling mice continued to show signs of illness but no death. In general, for the duration of the consecutive fourth through seventh (SB4-SB7) blind passages in suckling mice, no signs of illness were observed and no deaths due to illness occurred. However, SB5 passage on day two PI had one missing mouse, one dead mouse and one ruffled mouse. The ruffled mouse recovered on day three PI and showed no more signs before its termination on day seven PI. It is believed, since the death of the two mice were close to inoculation day and the ruffled mouse recovered and showed no more signs, that these observations resulted from complications during inoculation.

Material from SB1ASB1 was passed in one group of suckling mice (SB1ASB1SB1) and no death from illness and no signs of illness were observed.

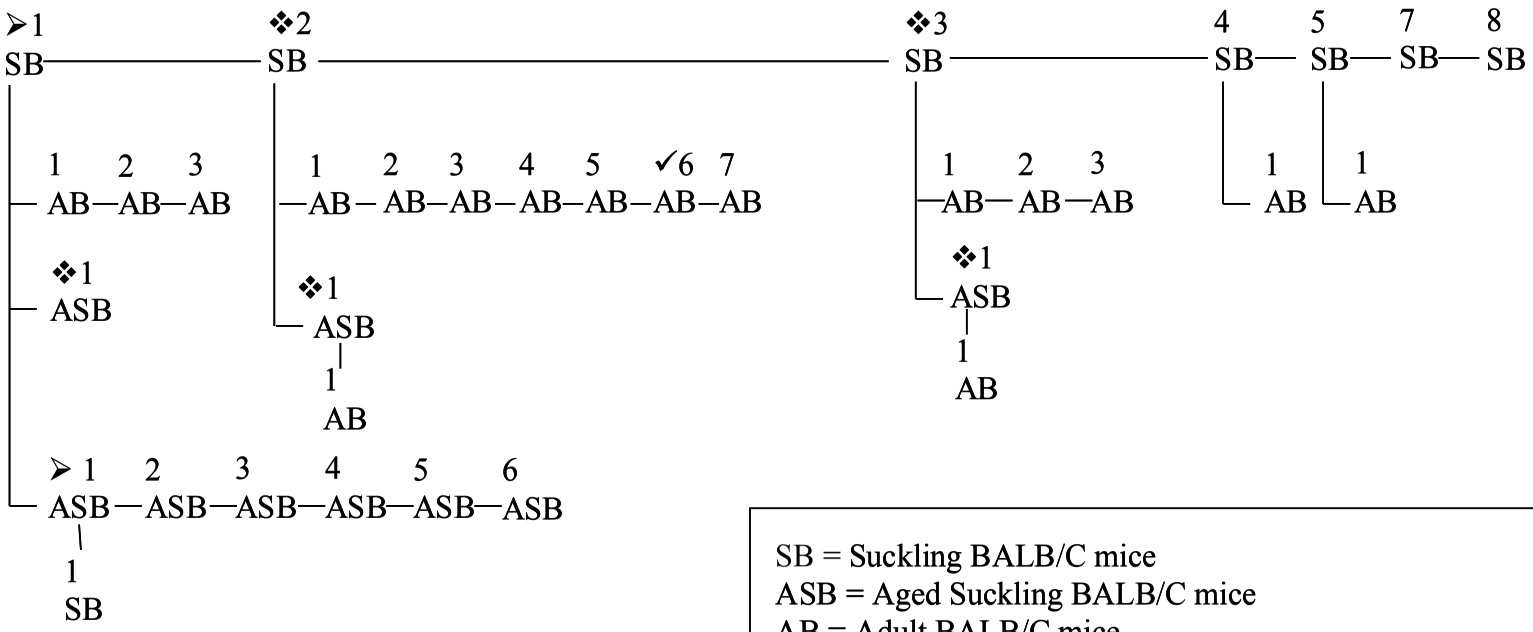


Figure 1. Experimental Mouse Passage Lineages

ASB Passages

Inoculation of SB1 material in ASB caused death and sickness in aged suckling mice (SB1ASB1), see Figure 1. Six of six mice became sick by day seven PI and three of those died on day eight PI. The three survivors continued to show signs for two more days through day ten PI and survived through day fourteen PI before being sacrificed. Day four PI passage material from SB1ASB1 group was passed in aged suckling mice for six consecutive blind passages (SB1ASB2-SB1ASB6), see Figure 1. No death from illness or any signs of illness were observed in these mice.

Inoculation of aged suckling mice (SB2ASB1) with SB2 material showed no death from illness but did show signs of illness. Two mice from SB2ASB1 appeared ruffled on day six PI and two on day seven PI. Both were consequently sacrificed on day seven PI.

SB3 material was passed in aged suckling mice (SB3ASB1) and no deaths occurred but mice did show signs of illness. Five of five aged suckling mice were perceived either ruffled or hunched on day six PI and on day seven PI two were ruffled/hunched and three had been eaten. SB3ASB1 material was passed in adult mice (SB3ASB1AB1) and no death from illness and no signs of illness were observed.

AB Passages

Upon initial passing of stock virus, no adult mouse died or showed any signs of illness due to infection. SB1 material was passed in a series of three AB (SB1AB1-3). No death from illness and no signs of illness were observed. SB2 material was passed in a series of seven adult mouse passages (SB2-AB1-7). No death from illness and no signs

of illness were observed. Passage of SB2ASB1 material into adult mice (SB2ASB1AB1) rendered no deaths and no signs of illness.

SB3 material was passed in a series of three consecutive adult mouse passages (SB3AB1-SB3AB3). Material from SB3ASB1 was also passed in adult mice. No death from illness and no signs of illness were observed in any of these passages.

Suckling mouse materials from SB4 and SB5 were each passed in adult mice (SB4AB1 and SB5AB). Neither passage had any deaths due to illness nor showed any signs of illness in any mouse.

Weight of AB Mice

All adult mice were weighed on a daily basis from pre-inoculation through day fourteen PI or until sacrificed. There was no significant weight loss in any of the mice due to Ebola infection. Since there were no signs of illness in any of the adult BALB/c mouse passages, this would be expected.

ELISA

Antigen detection by ELISA graph can be seen in Figure 2. In the SB group, 31 of 32 (10 of 10 day 4/5, 12 of 12 day 6/7, 4 of 4 day 8/9 and 5 of 6 day 14) tissue suspensions tested positive for EBO antigens by ELISA. The lone negative (SB2, day 14) was found in the second passage in SB mice (SB2). Although the pooled livers from SB2 day 14 are considered negative the results were close to the cut off value in titer and sum. The pooled spleens of SB2 mice were ELISA positive on day 14.

Pooled liver and spleen suspensions harvested on day 7 from SB2 were each passed in three different experiments in all three mouse groups (SB, ASB and AB). All SB

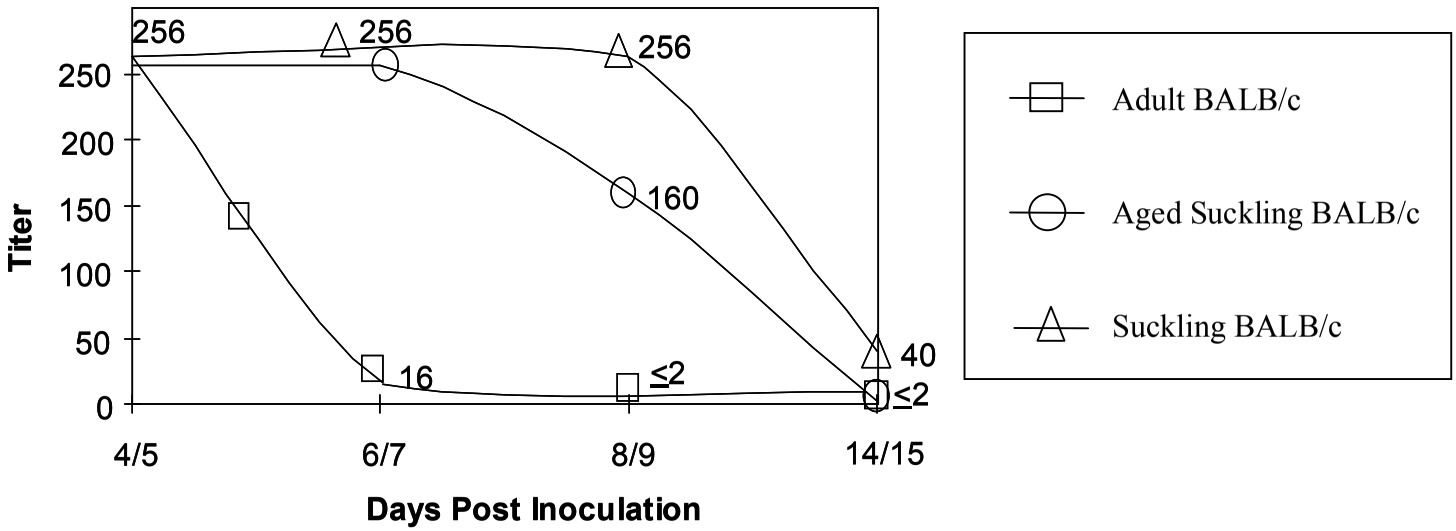


Figure 2. Median Ebola Antigen Detection Titers of BALB/c Mice after IP Inoculation of Blind Passaged Organ Suspensions

(day 7 and 14), AB (day 7), and ASB (day 4 and 6) groups receiving the inoculum from EMM19 day 7 liver and spleen passage material tested positive by ELISA.

In the ASB group, 23 of 23 suspensions tested from days 4 through 8, were positive for EBO antigen by ELISA. While all day 14 (16 of 16) suspensions tested were negative for EBO antigen by ELISA.

In the AB group from day 4/5, 30 of 35 were positive for EBO antigen by ELISA. The day 6/7 suspensions were similar with 13 of 18 testing positive for EBO antigen by ELISA. The day 8/9 and day 14 suspensions were significantly different in that only 5 of the 51 suspensions tested were positive for EBO antigen by ELISA.

Virus Titrations

Plaque assay results can be seen in Table 1. In the AB lineages, 2 of the 18 AB experimental passages had plaque producing suspensions. Each of the two separate passage suspensions were in the third passage (SB2-AB1) in AB, were duplicate experiments (20 and 21) and both plaque producing passage suspensions were from harvested spleens. No other AB suspension or suspension involving an AB linage produced plagues.

In the ASB lineages, 3 of 10 experimental passage suspensions produced plaques. Each of these suspensions was from harvested livers from consecutive blind passages (SB1-ASB4, 5 and 6) in ASB. Most notably the experiments were also the last three of the ASB's longest consecutive linage.

The SB lineages had 3 of 9 experimental passage suspensions produce plaques. Two were also in consecutive experiments (SB5 and SB6), as in the AB linage, and the third was from SB3 passage. Each suspension was from harvested spleen pools. The

Table 1 Plaque Assay Results for Mouse Passages

ADULT MICE			AGED SUCKLING MICE			SUCKLING MICE		
Tissue Tested[1]	**Passages in Mice[2]**	**Pos/Neg**	**Tissue Tested**	**Passages in Mice**	**Pos/Neg**	**Tissue Tested**	**Passages in Mice**	**Pos/Neg**
LV	2	-	LV	2	-	LV	1	-
SP	2	-	SP	2	-	SP	1	-
LV	3	-	LV	2	-	LV	2	-
SP	3	-	LV	3	-	SP	2	-
SP	3	+	LV	3	-	SP	3	-
LV	3	-	SP	3	-	LV	3	+
SP	3	+	LV	4	-	LV	3	-
LV	4	-	LV	4	-	SP	4	-
LV	4	-	LV	5	+	LV	5	+
LV	4	-	LV	6	+	LV	6	+
LV	4	-	LV	7	+	LV	7	-
LV	5	-						
LV	5	-						
LV	5	-						
LV	5	-						
LV	6	-						
LV	6	-						
LV	6	-						
LV	7	-						
LV	8	-						
LV	9	-						

[1]LV = liver tissue, SP = spleen tissue
[2]Number of times suspension was passed in mice.

suspension on SB3 was not involved in any further blind passages as a duplicate, simultaneously run, experiment was used for subsequent blind passing. The SB6 liver suspension was passaged one more time in SB and no plaques were produced.

CHAPTER 4

DISCUSSION

The majority of reports found indicated EBO-Z virus would infect suckling mice but not produce disease and death in adult mice (McCormick et al. 1983, Swanepoel et al. 1996). There was, however, one report of adaptation of EBO-Z in mice (Bray et al. 1998). In the study reported here we attempted to adapt a low passage EBO-Z strain to adult BALB/c mice.

The serial blind passages in mice followed a typical pattern of previous experiments. SB were found to be susceptible to lethal infection by EBO-Z as well as ASB while the AB did not show any signs of susceptibility. The same passage suspension that caused sickness and death in SB and ASB did not cause any illness or death in the AB passages. Looking at Fig 1, the linage shows that this pattern of illness and death in SB and ASB occurred on three occasions and passing these suspensions caused no illness or death in AB. The minimal changes in weights in the AB also support the no illness contention.

The antigen detection results indicate the suspensions that were passed in all of the groups had high antigen detection by ELISA (Figure 3) and that there was virus replication in all the mice regardless of age (Figure 4). The high antigen titers on day four for all passages tested, except for SB4 spleen, indicates that each mouse was infected and that the positive ELISA results were not from virus inoculum being carried from mouse to mouse but were a result viral replication. Antigen detection results do indicate

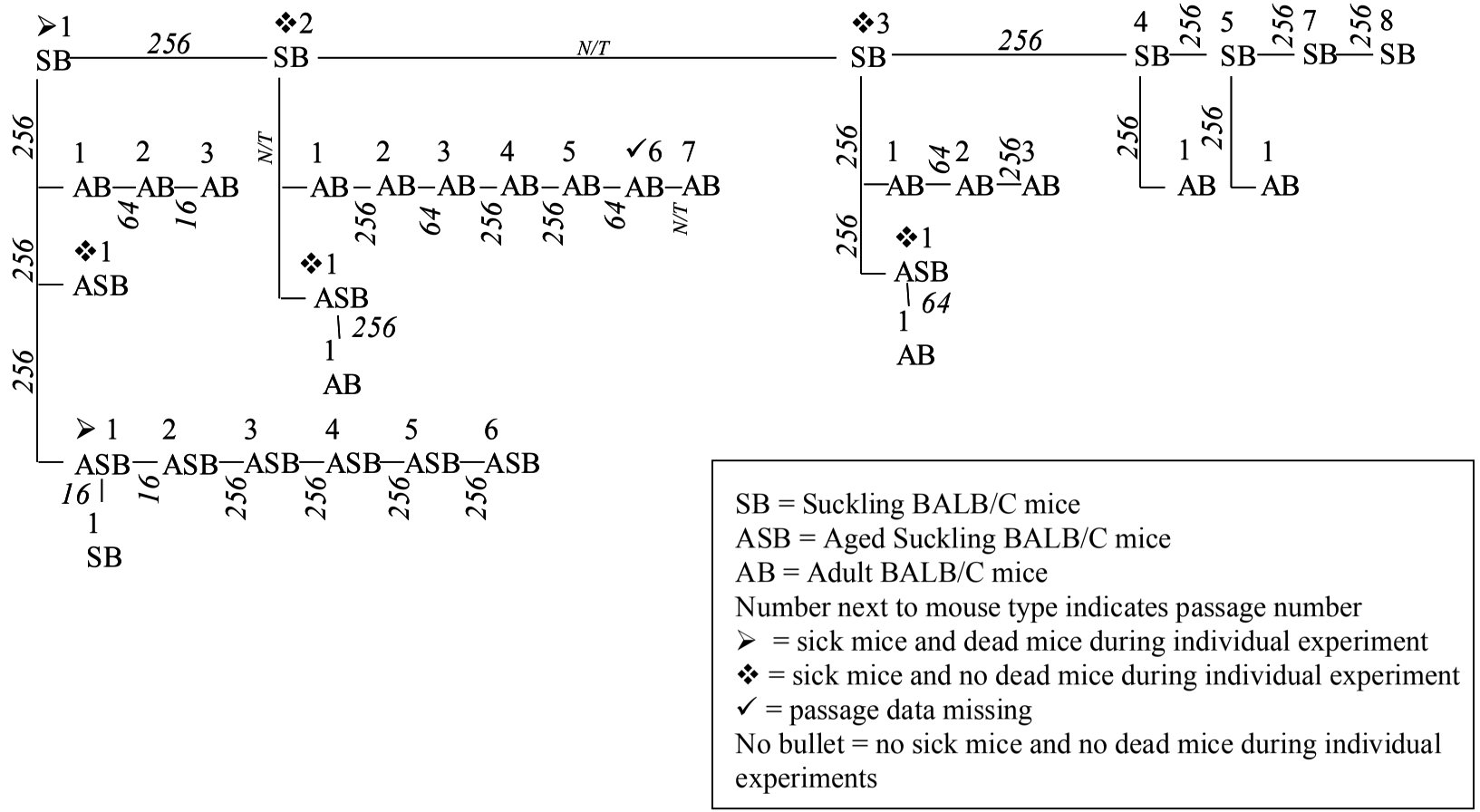
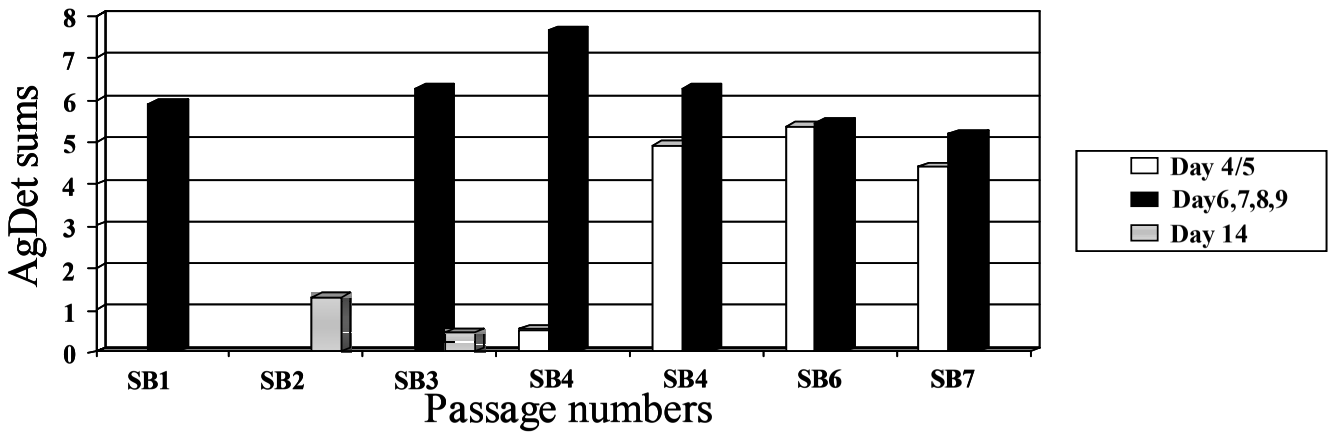


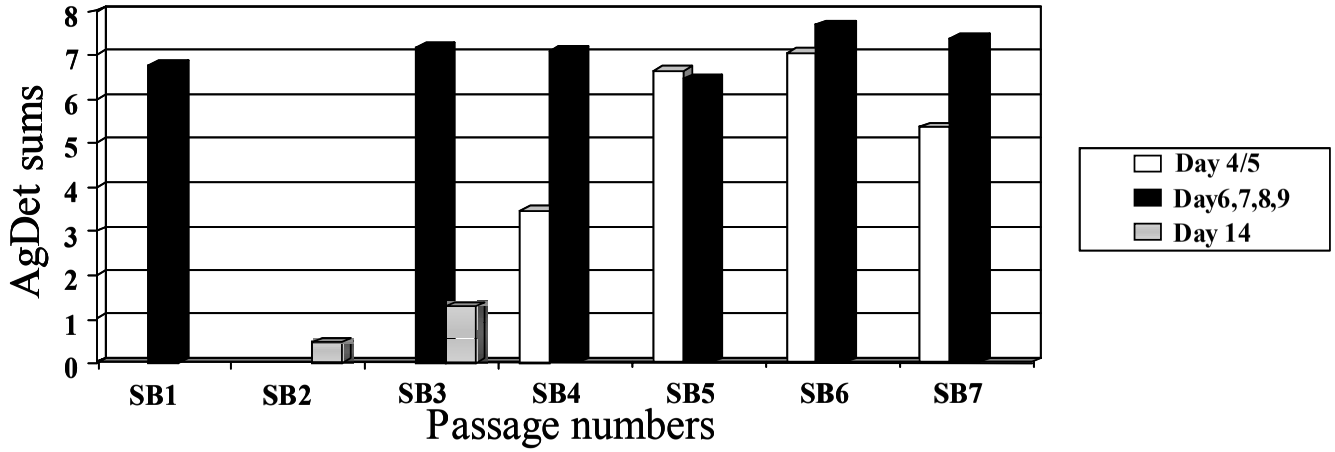
Figure 3. Antigen Detection Titer of Inoculum for Serial Blind Passages

Fig 4. Antigen detection by ELISA for various mouse lineages. Days indicate day post inoculation. AgDet sums are based on positive and negative titrations subtracted from each other and the four dilutions summed to reach a final OD reading. If multiple results were available for any passage material, the highest titer with the highest sum was used. (A) Blind passage of EBO Z LP through 8 consecutive blind passes in SB. (B) Passage of initial material harvest in SB1 passage and then passed in six consecutive ASB passages. (C) Passage of second SB passage material subsequently passed in seven consecutive AB passages.

Spleens



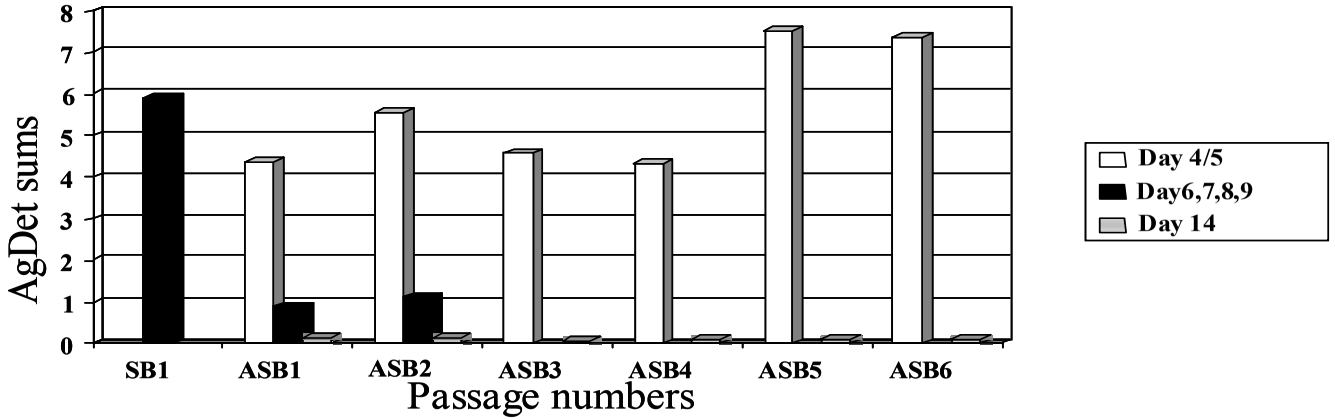
Livers



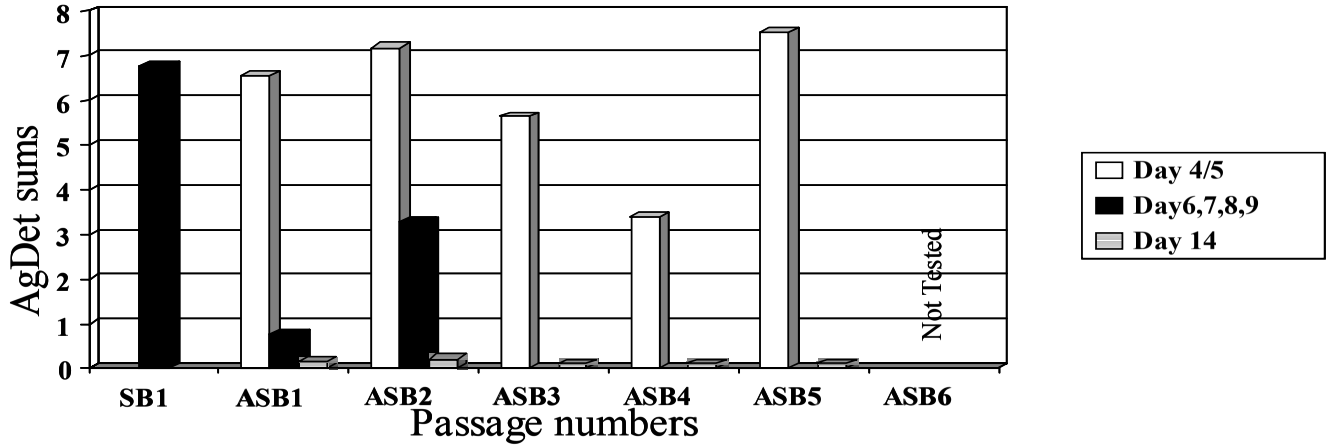
Note: If multiple results were available for any day, the higest titer was used.

Figure 4A. 7 Passes in SB AgDet results for Spleens and Lives

Spleens

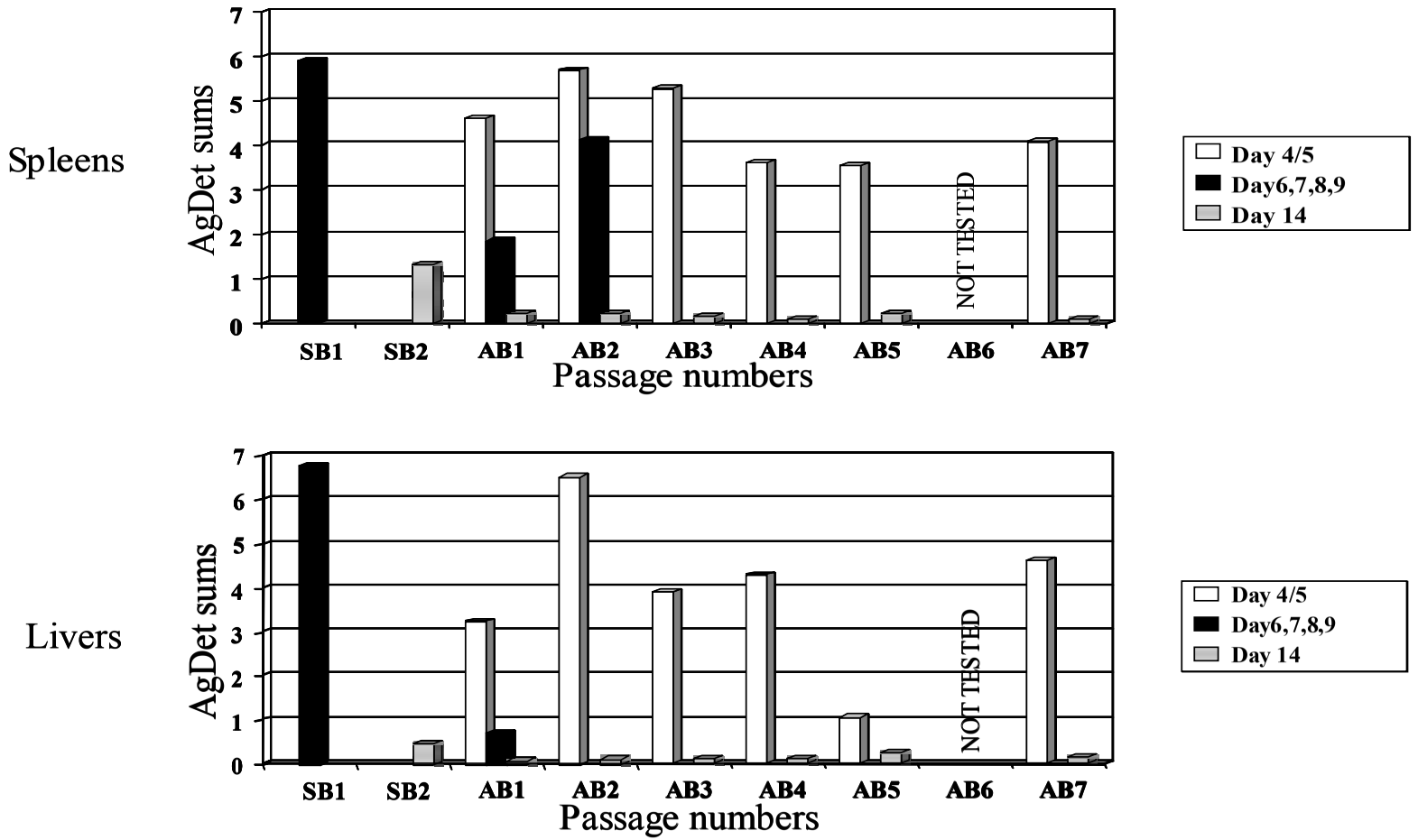


Livers



Note: If multiple results were available for any day, the higest titer was used.

Figure 4B. 1 Pass in SB and 6 Passes in ASB AgDet Results for Spleens and Livers



Note: Data for AB6 is missing. If multiple results were available for any day, the higest titer was used.

Figure 4C. 2 Passes in SB and 7 Passes in AB AgDet Results for Sleens and Livers

age does appear to be relevant in the ability of the mouse to clear the virus. AB and ASB showed a much more resilient ability to clear the virus as compared to SB (Figure 2). Of the 28 suspensions tested in AB only five were not antigen detection positive on Day 4/5 and two of those came from the same experiment and no mouse tested in that experiment became antigen positive indicating a possible inoculation problem. Discounting that experiment, all other experiments became antigen positive on or before day 6/7. Of those only two were still antigen positive on day 14. Similar results were noticed in the ASB as 6 of 6 suspensions tested on day 4/5 were positive for antigen and 10 of 10 suspensions tested on day 14 were negative for antigen by ELISA. The SB on the other hand had 5 of 6 suspensions positive on day 14. The deaths in the SB groups occurred on or before day 8/9 with no deaths occurring after day eight even though high antigen detection titers were found on day 14. These patterns were noticed early on in the experiment and most SB passages were harvested on or before day 8/9 to try to isolate as much antigen as possible. The resistance to EBO-Z infection in the maturing mice has been observed in previous experiments and it has been proposed that a type 1 IFN response may play a key role in the resistance of adult mice to EBO-Z infections (Bray 2001).

The antigen detection titers of the suspensions passed in this series of mouse passages appear to have no direct correlation with the ability of the suspensions to produce viable plaques. There were over twenty samples tested with an antigen detection titer of $\geq$ 256 and only three of these produced plaques. The highest plaque titer produced came from a suspension with an ELISA antigen detection titer of 1:64. The two AB passage suspensions that were antigen positive on day 15 did not produce plaques. The inability to produce plaques from antigen detection positive suspensions is a

phenomenon not completely understood. Perhaps there is a defective particle phenomenon happening. In the report on the adaptation of EBO-Z to adult mice, Bray et al. (1998) used a high passage virus plaquing technique to pick a specific plaque phenotype from Vero E6 cells to pass in mice. There was a significant change in the phenotype of the plaque picked from the suspension of passage eight of eight-day-old mice that led to the adapted strain. Bray et al. (1998) also noted that there was a 3 nucleotide difference in the GP sequence between the adapted stain and the original strain. These results strongly suggest the selection of a mutant sub strain of EBO-Z.

In the present study, with the low passaged EBO-Z strain, using the technique of enhancing the virus load by passage in suckling mice before passage into adult mice, we were unable to produce and active infection in adult mice. However, the data from the antigen detection by ELISA strongly suggests that there was virus replication in the adult mice early after inoculation (Figure 2, Figure 4). It is apparent from these observations that the AB is capable of mounting a protective immune response against EBO-Z. More recent experiments with the Bray strain have shown the ability of AB to artificially clear the Bray mouse adapted virus with the addition of immune serum which provides significant protection against lethal infection (Gupta et al. 2001). It is not understood whether or not this is achieved through a complete suppression of viral replication or by partial inhibition of replication. This demonstrated that in certain conditions mice treated with immune serum survived, cleared the virus and had no detectable viral antigen. Mice not treated with immune serum showed mortality and the titers of the suspensions tested for antigen detection by ELISA showed a direct correlation with the ability of these suspensions to produce viable plaques (personal correspondence). The mice in this study

survived, cleared the virus and had detectable viral antigen yet produced no viable plaques in Vero E6 cells. This suggests that there is significant viral replication carried on through out the blind passages and this replication can be measured by an ELISA technique but some how there may be enough partial inhibition of viral replication due to defective particles to inhibit replication in Vero E6 cells. Whether or not this is truly a result of a defective particles phenomenon is unknown.

It is believed that the ability to produce a viable mouse adapted EBO-Z strain is a chance occurrence that cannot always be reproduced. Consideration should be given to several alternative techniques with further attempts to produce a mouse adapted strain of EBO-Z. One technique could be to, again, use a plaque picked clone of the virus. This procedure would still depend upon picking a randomly mutated clone that would have increased virulence for mice. An alternative procedure would be to try a variety of inbred strains of mice. A costly procedure still dependent upon the chance match up of susceptible strain of mouse with the appropriate does of virus. Finally, immunosuppressive treatments could be used on adult mice. With serial passage in the compromised mice it may be possible to select an adapted strain of the virus.

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Appendix A

OPTIMIZATION OF NEW CAPTURE AB
IN IMMUNOCAPTURE ASSAYS
13 JULY 1990
KSIAZEK, THOMAS G

1 Safety: The controls and antisera used in the performance of this assay have been treated to inactivate any of the new virus that may have been in them at the time of production. Within the limits of our ability to detect viable virus these products are safe. The material to be tested for the presence of IgG for the new viral agent is potentially contaminated with viable new virus or other agents for which a differential determination is being sought. Material collected from human sources is potentially contaminated with human viruses such as hepatitis B or HIV. Accordingly, caution should be exercised in handling all materials associated with this test. If hazard is high, work should be conducted in an appropriate containment environment. Good laboratory practices at the P2 level should be used at minimum.

2 Background: In developing a new antigen detection assay or changing lots of antibody in an existing assay it is necessary to make certain that one is using optimal concentrations of the new reagents to assure that the new reagent lots perform at optimal levels or that they perform as well the previously used lot.

3 Experimental Protocol: This SOP describes the general procedure for determining the optimal dilution of capture antibody to be used in an assay. The procedure is basically a cross-titration (or checker board titration) of capture antibody and

a positive and negative control antigen (or several of each). One evaluates the outcome by seeking the capture antibody dilution which yields the maximum OD values at the extreme dilutions of the positive control antigens. It is important to use a positive antigen (or terminal dilutions of the antigens) which does not "max" the OD values throughout the range of dilutions used in the test.

4 Methods and Materials

a PBS: Phosphate buffered saline (0.01 M, pH 7.4). Use Sigma 1 liter packs (Sigma Cat. No.1000-3). May add thimersol to 1:10,000 concentration.

b Wash buffer: PBS with Tween-20 (0.1%), pH 7.4, with or without thimersol as a preservative.

c Serum Diluent: Wash buffer with protein blocking buffer: most commonly skim milk but one should check the protocol for the assay for which you are titrating a new lot of antigen and use the SerDil as described in the SOP for that assay.

d Plates: PVC, Dynatech Cat. No. 001-010-2101.

e Capture antibody: Anti-virus MAb, HMAF, or other serum used in existing SOP or that proposed to be used in a system under development.

f Coating conditions for capture antibody: Diluted 1:200--> 2-fold, down plate. Diluted in PBS (or rarely another buffer system) down with no tween, pH 7.4 and coated overnight at 4C.

g Antigen and Control Antigen:

i Positive antigen, diluted 1:X-->2-fold, across plate.

ii Negative antigen, diluted 1:X-->2-fold, across plate.

h Positive and negative detection antibody (sera or fluids) are diluted 1:1000 (usually a good starting point) in SerDil and tested against both positive and control antigens.

i Conjugate: A conjugate directed at the species of the detection antibodies is used. The dilution used must be based on previous experience with the conjugate in the assay for which a new Ag is being titrated or upon experience in similar assays when a new test is being assembled. If a new assay is being developed, the ultimate conjugate dilution will be based on a subsequent optimization procedure.

j Substrate: ABTS (Kirkegaard and Perry, Cat. No.506201). Parts A & B combined 1:1 immediately before use.

5 Interpretation of results:

a Again one needs to view several parameters in the interpretation of the assay.

i The values of the negative antigen at each dilution is subtracted from the positive antigen. This remainder, or the adjusted OD for each dilution will yield a pattern that will give the dilution at which the capture antibody has maximum capture ability.

ii The optimal dilution is generally considered to be one dilution below that at which a "shoulder" or decrease in the marginal adjusted OD values occurs. In the antigen detection assay, these adjusted OD values should usually be quite high at the lower dilutions of the positive antigen.

6 Flow chart. (all volumes=100 microliters)

Coat plate with anti-viral capture antibody at 1:200-->(2-fold, down)

in PBS (no tween), pH 7.4, overnight, 4C.

|

Washing 3X

|

Add Pos Antigens (1:X-->2-fold across) in SerDil

(Neg control antigen, same dilutions)

Incubate 60 min 37C

|

Wash 3X

|

Add positive and negative detection sera

1:1000 (probably a good first guess) in SerDil

Incubate 60 min 37C

|

Wash 3X

|

Add K&P ABTS substrate

Incubate 30 min at 37C.

|

Read at 410 and 490 nm

Appendix B

OPTIMIZATION OF NEW DETECTOR AB
IN IMMUNOCAPTURE ASSAYS
13 JULY 1990
KSIAZEK, THOMAS G.

1 Safety: The controls and antisera used in the performance of this assay have been treated to inactivate any of the new virus that may have been in them at the time of production. Within the limits of our ability to detect viable virus these products are safe. The material to be tested for the presence of IgG for the new viral agent is potentially contaminated with viable new virus or other agents for which a differential determination is being sought. Material collected from human sources is potentially contaminated with human viruses such as hepatitis B or HIV. Accordingly, caution should be exercised in handling all materials associated with this test. If hazard is high, work should be conducted in an appropriate containment environment. Good laboratory practices at the P2 level should be used at minimum.

2 Background: In developing a new antigen detection assay or changing lots of antibody in an existing assay it is necessary to make certain that one is using optimal concentrations of the new reagents to assure that the new reagent lots perform at optimal levels or that they perform as well the previously used lot.

3 Experimental Protocol: This SOP describes the general procedure for determining the optimal dilution of detection antibody to be used in an assay. It follows a previous experiment in which the dilution at which the capture antibody has been determined (alternatively, this can be done by simultaneously doing a large experiment in

which both reagents are diluted by doing separate microplates, each with serial dilutions of the detection antibody). The procedure is basically a cross-titration (or checker board titration) of detection antibody and a positive and negative control antigen (or several of each). One evaluates the outcome by seeking the capture antibody dilution which yields the maximum OD values at the extreme dilutions of the positive control antigens. It is important to use a positive antigen (or terminal dilutions of the antigens) which does not "max" the OD values throughout the range of dilutions used in the test.

4 Methods and Materials:

a PBS: Phosphate buffered saline (0.01 M, pH 7.4). Use Sigma 1 liter packs (Sigma Cat. No. 1000-3). May add thimersol to 1:10,000 concentration.

b Wash buffer: PBS with Tween-20 (0.1%), pH 7.4, with or without thimersol as preservative.

c Serum Diluent: Wash buffer with protein blocking buffer: most commonly skim milk but one should check the protocol for the assay for which you are titrating a new lot of antigen and use the SerDil as described in the SOP for that assay.

d Plates: PVC, Dynatech Cat. No. 001-010-2101.

e Capture antibody: Anti-virus MAb, HMAF, or other serum used in existing SOP or that proposed to be used in a system under development.

fii Coating conditions for capture antibody: Diluted at the optimal dilution found in the previous checker-board optimization. Diluted in PBS (or rarely another buffer system) down with no tween, pH 7.4 and coated overnight at 4C.

g Antigen and Control Antigen:

iii Positive antigen, diluted 1:X-->2-fold, across plate.

iv Negative antigen, diluted 1:X-->2-fold, across plate.

h Positive and negative detection antibody (sera or fluids) are diluted from 1:250 (usually a good starting point) in 2-fold dilutions in SerDil and tested against both positive and control antigens.

i Conjugate: A conjugate directed at the species of the detection antibodies is used. The dilution used must be based on previous experience with the conjugate in the assay for which a new Ag is being titrated or upon experience in similar assays when a new test is being assembled. If a new assay is being developed, the ultimate conjugate dilution will be based on a subsequent optimization procedure.

j Substrate: ABTS (Kirkegaard and Perry, Cat. No. 506201). Parts A & B combined 1:1 immediately before use.

5 Interpretation of results:

a Again one needs to view several parameters in the interpretation of the assay.

i The values of the negative antigen at each dilution are subtracted from the positive antigen. This remainder, or the adjusted OD for each dilution, will yield a pattern that will give the dilution at which the detection antibody has maximum detection ability.

ii The optimal dilution is generally considered to be one dilution below that at which a "shoulder" or decrease in the marginal adjusted OD

values occurs. In the antigen detection assay, these adjusted OD values should usually be quite high at the lower dilutions of the positive antigen.

b This may be followed with a third assay in which the conjugate which detects the detector antibody is optimized by cross-titration of the conjugate with the detector antibody while the capture antibody and the antigen are held constant.

6 Flow chart. (all volumes=100 microliters)

Coat plate with anti-viral capture antibody at optimal dilutions (previous assay)

in PBS (no tween), pH 7.4, overnight, 4C.

|

Washing 3X

|

Add Pos Antigens (1:X-->2-fold across) in SerDil

(Neg control antigen, same dilutions)

Incubate 60 min 37C

|

Wash 3X

|

Add positive and negative (separate plates) detection sera

1:250B> in 2-fold steps in SerDil

Incubate 60 min 37C

|

Wash 3X

|

Add K&P ABTS substrate

Incubate 30 min at 37C.

|

Read at 410 and 490nm