PRODUCTION OF MONOCLONAL ANTIBODIES TO THE HN GLYCOPROTEIN OF THE OSU-T STRAIN OF SENDAI VIRUS AND THEIR USE IN PRELIMINARY STUDIES OF VIRAL PENETRATION OF CELLS

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PREFACE

Hybridoma cells producing monoclonal antibodies to the HN glycoprotein of the OSU-T strain of Sendai virus were developed and used in preliminary antibody blocking assays. Though the actual procedures involved in hybridoma techniques are not difficult, finding precise procedures that would work in our laboratory took time and quite a bit of trial and error. Even when all the procedures are successful, the time from the first immunization of the mice to the time the antibodies are in hand takes at the minimum three to four months. In addition, liposome assays which indicated that Sendai virus perturbs membranes so that molecules as large as an antibody molecule can escape were done.

Because the opportunities for discouragement were so great during this project, I would especially wish to thank my major advisor, Dr. Mark R. Sanborn, for his unending optimism and enthusiasm, as well as for his guidance and encouragement. I would also like to thank Dr. James Harmon for his many helpful suggestions, Dr. Helen Vishniac, and everyone else who assisted me in this work at Oklahoma State University.

Special thanks goes to Martha Sheriff who patiently typed this manuscript and to my husband, John, who never let me quit.

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CHAPTER I

INTRODUCTION

The general replication cycle for all viruses is currently given as attachment, penetration, uncoating, replication, maturation, and release. Two general but opposing theories have been presented for the penetration and uncoating of Sendai virus (Parainfluenza 1): fusion and viropexis. Fusion has been proposed by many, and in fact, this mechanism is portrayed as the major mode of entry in textbooks (16).

Fusion as a hypothesis for penetration and uncoating of the virus is supported by electron microscope studies (40), evidence that viral components become randomly distributed on cell membranes (68), and the ability of Sendai virus to cause cell - cell fusion (40). However, studies using liposomes as artificial membranes indicate that the virus penetrates by viropexis (24) with concomitant perturbation of the host cell membrane. This view is also supported by research done in our laboratory.

The purpose of this research has been to produce monoclonal antibodies to Sendai virus and to use these antibodies in preliminary studies examining the processes

of penetration and uncoating in Sendai virus. A model system of antibody loaded liposomes was used. In this system, if viral membrane - liposomes membrane fusion occurred upon infection by Sendai virus, the antibody inside the liposome membrane would be unable to escape. However, if the liposome membrane were perturbed or broken, the antibody inside would escape and be detectable by the Enzyme Linked Immunosorbent Assay (ELISA). In addition, monoclonal antibodies were used in preliminary experiments to try to block the action of the virus. The results of these experiments indicate that antibody does escape from the loaded liposomes upon infection by Sendai virus but that anti - HN antibody does not block infection under the preliminary experimental conditions used.

CHAPTER II

REVIEW OF THE LITERATURE

History

In 1952, Kuroya et al. isolated a previously undescribed infectious agent from an epidemic of fatal pneumonitis in newborn children. After attempts to cultivate the agent *in vitro* were unsuccessful, mice inoculated with the agent developed influenza-like lesions of the lung. Fluids collected from infected eggs caused agglutination of chicken red blood cells. The agent was designated newborn pneumonitis virus, type Sendai, when attempts to identify it as a known virus failed (51). Other strains of Sendai have originated from mice or hamsters (33).

In 1955, a possible relationship between Sendai virus and the group of viruses containing mumps, Newcastle Disease virus (NDV), influenza, and fowl plague was suggested (39). Sendai virus was not included when a description of these viruses, the myxoviruses, appeared in the same year (2). However, in 1959, the name myxovirus parainfluenza 1 was proposed for Sendai virus because of its resemblance to mumps and NDV in its ability to hemolyse and agglutinate certain erythrocytes (3). When

Sendai virus and other newer myxoviruses HA-1 (hem-adsorption), HA-2, and CA (croup-associated) were studied on the basis of their antigenic relationships, it was found that these viruses were unrelated antigenically to Influenza viruses types A, B, and C. No one common antigen was found between the four new myxoviruses and NDV, but each one was found to be related to one or more of the others, with the exception of HA-2 which was found to be a subgroup of Sendai (9). In 1962, the myxoviruses were divided into two subgroups, orthomyxoviruses and paramyxoviruses, with influenza virus and NDV being the representative viruses (92). In 1973, Sendai was placed in the paramyxovirus group (59).

Morphology

Sendai virions have been shown to be spherical in shape, with diameters ranging from 100 to 600 nm (30). This pleomorphism has been reported to be due to the occurrence of virions with polynucleocapsids (32). Sendai virus was once thought to contain a rigid double-stranded RNA structure (88), but was later found to be a negative-sense single-stranded helix with 11 to 13 subunits in each turn (14), 18 nm wide (30), and 1 um long (31). Virion RNA was found to self anneal (77) as a result of its content of complementary strands. The molecular weight of the virion RNA was found to be 6×10^6 (46). In addition to the helix, spikes with lipotrophic properties were described (20).

In 1971, an RNA dependent RNA polymerase was found in nucleocapsid-like structures also known as transcriptive complexes (58) (80). These structures, which transcribe genome RNA within the nucleocapsid (5), contain two polypeptides. The major component of the nucleocapsid, NP, was found to have a molecular weight of 60,000 (58) (97). P, the largest protein, was found to have a molecular weight of 75,000 (58) (97). The least abundant molecule of the nucleocapsid, L (54), has often been ignored. One source has given the molecular weight of L as 75,000 (87). In 1974, the smallest virion protein, M, molecular weight 35,000 was described as the membrane or matrix protein (96). The function of M has been suggested as associating the nucleocapsid to the envelope (95). The NP, P, and M proteins have been found to be phosphorylated (52). L, P, and NP were determined to be internal (4), while M was found to be a peripheral protein (6). In addition to the L, P, NP, and M proteins, two glycoproteins have been identified as well as a pair of non-structural proteins, C (53) and C' (18) (53).

The Glycoproteins

Two distinct types of Sendai virus spikes have been isolated with both being described as dumbbell in shape. The larger glycoprotein (HN) was found to contain both hemagglutinin and neuraminidase activities and to have a molecular weight of 67,000 (86). The other glycoprotein,

designated F, has been found to consist of two glycoproteins, F_1 and F_2, with molecular weights of 51,000 and 11,000 (17). These glycoproteins were found to be linked by a disulfide bond. Cleavage of this bond was found to be a prerequisite for activity (67), and was found to cause a conformational change with an increase of exposed hydrophobic surface (34) on the amino terminus. The amino acid sequence of this terminus has been determined (17) and it is thought to interact with the cell membrane (79), although the exact nature of this interaction has not been determined. It has been suggested that the carboxy terminal region is associated with the viral membrane (79).

Fusion of Sendai Virus With Cells

Role of HN

By using immunological methods, Merz, et. al. found that antibodies against the HN glycoprotein blocked virus adsorption (60) a property attributed to the HN glycoprotein because of the receptor-binding function of the hemagglutinin (82). In addition, they found anti-HN antibodies caused inhibition of hemolysis (61) when added before adsorption of virus to cells.

Ozawa et. al. (74) examined the role of the HN glycoprotein using two different approaches. The HN glycoprotein was denatured by dithiothreitol and the lectin, concanavalin A, was used to replace the lost

receptor-binding function. In the other approach, influenza virus HA glycoprotein and NA glycoprotein were mixed with dithiothreitol-treated Sendai virus forming a hybrid envelope. Erythrocytes were infected with the two virus preparations and hemolysis of the red blood cells used as a measure of fusion. In both cases hemagglutination did not decrease yet, since hemolytic activity was absent, the researchers concluded that the HN glycoprotein of Sendai virus is required for virus-cell fusion in a role other than binding of the virus to the cell. Gitman et. al. (19) have also suggested that the HN glycoprotein has an active role in fusion after finding that only reconstituted Sendai virus envelopes that contained untreated viral glycoproteins were fusogenic.

Markwell et. al. (57) however, found that a Sendai virus mutant (HN- when grown at 38°C) was able to cause membrane fusion in human hepatoma Hep G2 cells even though it was unable to infect or agglutinate conventional host cells: MDBK, HeLa, and MDCK cells. These researchers found that the F glycoprotein could serve as its own attachment protein due to its ability to bind to the asialo-glycoprotein receptor present on Hep G2 cells. They concluded, then, that the HN glycoprotein role in fusion is limited to attachment and that the discrepancy between their results and the previous conclusions resulted from the fact that in the previous studies model

systems were used in which the HN glycoprotein was destroyed by dithiothreitol.

Peterhans et al. (75) also supported the idea that F has its own receptor site and the HN and F interact with the cell membrane independently. They based their conclusions on studies in which they measured chemiluminescence when liposomes containing either purified HN or F were added to mouse spleen cells.

Although a definite role for neuraminidase has not been established, neuraminidase activity has been shown to be inhibited by anti-HN antibodies (72). It has been suggested that neuraminidase may be involved in releasing the virus from cells (25) (69). Huang et. al. have stated that a neuraminidase is required for fusion to occur and argue that an enzymatic role and not a structural role is involved (36). Thess suggestions have been questioned by Markwell, et. al. (57) and Hsu et. al. (35) who have stated that neuraminidase is not required for fusion. Kohama, et. al., when finding no detectable amount of sialic acids in the Sendai virion itself, implied that the role of the neuraminidase is to remove any sialic acids in the virus (44) thus preventing the aggregation of newly-released viruses (8). Using monoclonal antibodies to the HN glycoprotein, Portner has suggested that possibly the hemagglutinin and neuraminidase may involve independent sites rather than a single active site (76).

Role of F

Before the use of immunological studies or the reconstitution of membranes containing purified viral glycoproteins, Scheid et. al. postulated that because the HN glycoprotein contained the hemagglutinin and neuraminidase activities, the F glycoprotein must possess the other activities associated with the virus; cell fusion and hemolysis (82). Later Scheid and others, as well as Homma and Ohuchi (28) found that Sendai virus grown in cells contained the uncleaved form of F and did not induce infection or cause cell fusion or hemolysis. On the other hand, egg grown Sendai virus, possessing all of these activities, contained the cleaved form of F necessary for infection (28) (83) (84). Thus, Choppin and Scheid have implied that because adsorption occurs regardless of whether F is cleaved and because both infectivity and fusion [hemolysis has been stated as involving fusion (41) (64) although this has been disputed (50)] do not occur unless the F protein is cleaved then the "mechanism of penetration of paramyxoviruses is fusion of the viral and cell membranes" (8).

Throughout the years, many scientists have studied the entry of Sendai virus into cells. In almost all the reports, different conditions including various cell lines or primary cells, different virus preparations, and different methods of entry measurement were used. As early as 1968, Morgan and Howe observed fusion of virus

with either chorioallantoic membrane or L cells using the electron microscope (64). As previously mentioned, Scheid and Choppin looked at cleaved versus non-cleaved F and used cell-to-cell fusion of BHK-21 cells as a measure of virus entry (83). Miyake et. al., however, were able to separate viral envelope-cell membrane fusion and cell-cell fusion. They found that cytochalasin D inhibits cell-cell fusion but not virus-cell fusion (63). Fan and Sefton (13) took another approach in determining the mechanism of entry into cells by Sendai virus. They reasoned that if Sendai virus entered by fusion, then viral antigens would remain on the surface of the cell and the cell could be killed by the addition of anti-Sendai antibody and complement. Using MDBK cells prelabeled with ^{51}Cr-sodium chromate that were newly infected with either active or inactivated Sendai virus, Fan and Sefton measured the amount of label released when the cells lysed after the addition of antibody and complement. They reported that their results support that Sendai virus enters cells via fusion.

Knutton wrote a series of articles examining Sendai virus fusion of erythrocytes and HeLa cells with the scanning electron microscope (40) (41) (43). In these studies, Knutton also approached the problem of virus-cell fusion by examining Sendai virus induced cell-cell fusion. In his model, the viral envelope would fuse with the cell membrane through two cell-mediated invaginations

on the viral envelope which would become incorporated into the cell membrane. Then, due to virally-induced cell swelling, the entire viral envelope would become a part of the cell membrane. Cell to cell fusion would occur then, when a single virus particle fused with two cells (42). This crosslinking has been termed bridging and there has been no morphological evidence that this mode exists (42) (33). Knutton, having demonstrated endocytosis of intact virus particles states than even though endocytosis occurs, the very existence of the specialized cell-mediated mechanism for fusion implies that fusion rather than endocytosis is the mechanism of entry for Sendai virus (42).

Knutton further supported his model by using results provided by Homma et. al. (29) which stated that early harvest virus "1-day" unlike "3-day" virus, while still possessing infectivity and cell fusion activity, did not possess hemolytic activity. However, freezing and thawing or sonicating 1-day virus restored hemolytic activity. Knutton compared the fusion of 1-day virus with erythrocytes and 3-day virus with erythrocytes and determined that "cell-swelling" which occurred only with the 3-day virus was the "driving force" for the incorporation of the viral envelope into the cell membrane. He reported that without this cell swelling, lateral diffusion of the viral envelope components did not occur. Also, the cell swelling allowed the formation of

spherical fused cells. Impraim, et. al. (37) have found that sometimes 3-day virus has 1-day virus like character and therefore, they have concluded that a variable other than growth time affects the virus' ability to cause permeability changes.

Knutton, together with Wyke et. al. provided further information on permeability changes caused by Sendai virus (93). They measured leakage of [^{3}H] choline-derived metabolites from prelabeled Lettree cells infected with enzymatically modified Sendai virus (either HN- or F-) and found that the presence of the F glycoprotein affected permeability changes more than did the presence of the HN glycoprotein. In addition, they found that fluorescently labeled peptides with a molecular weight greater than 1000 did not enter infected cells easily. Based on these observations, they estimated that a pore existed of approximately 1 nm in diameter. They also provided evidence agreeing with Volsky and Loyter (91) that virus-cell fusion is independent of Ca^{++} concentration, although Impraim, et. al. (37) reported that Ca^{++} inhibits permeability changes. Foster, et. al. found that Sendai virus causes permeability changes in many different cell types (15).

Using virus loaded with the spin probe Tempocholine, Maeda et. al. (56) used two methods to study its release from the virus and found that Tempocholine was rapidly released when the virus interacted with erythrocytes and

that active F was required. Using Knutton's model, they proposed that following the cell's increased permeability a certain threshold in the amount of water entering or in the cell's volume is reached and an enhancement of phospholipid transfer between virus and cell occurs.

Using a different approach, Israel et. al. (56) proposed that a virus-associated protease may be involved in virus-cell fusion. They based this on work that supported the presence of a virus-associated protease which could hydrolyze certain polypeptides and could be inhibited by inhibitors of proteolytic enzymes. These inhibitors also inhibited the hemolytic and fusogenic activities of the virus, but not its ability to agglutinate erythrocytes. The same results were seen with reconstituted Sendai virus envelopes, indicating an association of the protease with the viral glycoproteins. They proposed that the protease hydrolyzes glycoproteins on the cell surface so that certain membrane phospholipids are then exposed to the F glycoprotein and fusion can occur.

Other studies have examined the role of the F glycoprotein in fusion using viral preparations other than whole virus and/or using liposomes rather than cells. In 1974, Haywood (22) (23) first reported the fusion of Sendai virus with liposomes and a series of research papers followed. Haywood reported that both fusion and a step resembling phagocytosis of the virus occurred

depending upon the composition of the liposome (24). Haywood and Boyer studied conditions to optimize fusion of Sendai and liposomes (26) and stated that optimal conditions are the same as for fusion of the virus with host cells and red blood cells. Haywood and Boyer have also proposed a model for fusion (25). In this model, fusion occurs at the "leading edge" of what appears to be the cell engulfing the virus and the viral membrane unwinds until it is a part of the cell membrane. Recently, Haywood and Boyer looked at lipid composition of liposomes and the effect on fusion with Sendai virus (27). They concluded that the virus membrane can fuse with negatively charged liposomes and that the presence of cholesterol is not necessary. They have stated that their conclusions support that fusion is dependent upon the viral proteins rather than lipid composition.

Haywood and Boyer (27) strongly disagreed with work done by Hsu et. al. (35) who stated that the presence of cholesterol in liposomes was required for fusion. Hsu et. al. used sonicated liposomes in two methods designed to observe fusion of the liposomes by Sendai virus. In the first method they separated fused liposomes from unfused liposomes by centrifugation and in the second method, they digested internal viral proteins after fusion with trypsin loaded liposomes. Haywood and Boyer disputed both these methods for several reasons including the belief that Hsu et. al. were measuring something other than fusion.

Ozawa and Asano (73) used a different approach to look at factors required for fusion. They formed liposomes containing pure HN and F glycoproteins in either defined lipids or lipids extracted from the virus and used these liposomes to induce cell fusion of both Ehrlich tumor cells and erythrocytes. They found that F must be present for fusion and for hemolysis but not for hemagglutination. They also reported that no viral glycolipids were needed for fusion although they found that a high cholesterol content was needed in each fusing membrane, whether those membranes were viral and cellular or of two cells.

Kruse et. al. (50) isolated glycoproteins that were not incorporated into a lipid bilayer to study fusion. They found that their preparation fused cells as well as whole virus did. Their preparation was partially lipid depleted and contained a higher cholesterol to lipid ratio than did whole virus. They concluded that the fusogenic activity lies with the glycoproteins alone.

Lee et. al. (55) looked at rotational mobility of the glycoproteins to see if it had some connection to fusion activity. They concluded that the mobility of the glycoproteins appeared to be correlated with fusion activity but not with hemagglutination activity.

Abidi and Yeagle (1) used ^{31}P nuclear magnetic resonance to study the viral glycoproteins. They found that when trypsin was used to eliminate the F

glycoprotein, the highly restricted phospholipid headgroup environment was relieved. They suggested that F is involved in tightly organizing the viral membrane.

Chejanovsky, et. al. (7) incorporated chlorophyll a and chlorophyll b into reconstituted Sendai virus particles and measured energy transfer between the two chlorophyll molecules as an indication of fusion of the virus particles with erythrocytes and Ehrlich ascites tumor cells. They found that the efficiency of energy transfer decreased upon fusion as compared to the virus particles alone. This decrease was not seen when non-fusogenic virus particles were used.

Oku, et. al. (70) found that Sendai virus fused with liposomes affected permeability as was found with cells.

Tsao and Huang (89) loaded liposomes with calcein and found that leakage occurred when the liposomes were infected with intact Sendai virus, but this leakage was not apparent when liposomes were infected with trypsin-treated virus (F-). They also found that the presence of cholesterol in their liposomes suppressed leakage. They speculated that leakage is due to fusion of the virus with the liposomes.

Monoclonal Antibodies to Sendai Virus

In 1975, Kohler and Milstein (45) reported a technique that allowed for the fusion of mouse myeloma cells and mouse spleen cells from an immunized donor, which led to

hybridoma cell lines that produced specific antibody directed against a predetermined antigen. Two years later, Koprowski et. al. (47) showed that it was possible to produce monoclonal antibodies to specific viruses and their antigenic determinants. This technique was used to study antigenic drifts in viruses, including Sendai virus (78). In 1981, monoclonal antibodies to the HN glycoprotein of 6/94 virus, a parainfluenza type 1 virus, related to Sendai virus, were made (94). Two kinds of monoclonal antibodies to the HN glycoprotein of Sendai were isolated by Miura et. al. (62). They reported that one antibody inhibited hemagglutination and neuraminidase activities, and that the other inhibited virus-cell fusion, cell-cell fusion, and hemolysis (62). Orvell and Grandien (71) reported the production of monoclonal antibodies to the NP, P, and M proteins, as well as to the F and HN glycoproteins. They used these antibodies to study the appearance of the viral antigens in infected Vero cells. These monoclonal antibodies were also used to study the presence of viral antigens within cells (48) and to look at viral infection in mouse brain (49). Sato et. al. used monoclonal antibodies to the HN glycoprotein to examine persistant viral infection of cells by Sendai virus (81). Most recently, Deshpande and Portner have used monoclonal antibodies to the NP (11) and to the P (12) proteins to study the structure of the proteins and to find information concerning their roles in transcription.

The purpose of this research has been to begin a monoclonal antibody bank to Sendai virus proteins which would aid in the study of viral penetration. The antibodies could be used in examining both the OSU-T strain and the type strain of Sendai virus and in preliminary experiments examining the process of viral penetration.

CHAPTER III

MATERIALS AND METHODS

Water and Autoclave

Water used in all preparations and experiments was filtered through a NANOpure system (Barnstead). All autoclaving was done in a Barnstead laboratory Sterilizer using NANOpure water. All solutions and materials were autoclaved at 121°C, 15 pounds of pressure, for a minimum of 15 minutes unless otherwise noted.

Virus Stock

The OSU T strain of Sendai virus was propagated by injecting one hemagglutinating unit (HAU) of virus per egg into ten day old embryonated chicken eggs. The eggs were incubated at 31°C for 48 hours and removed to 4°C for 2 to 18 hours before the allantoic fluid was collected. To precipitate the virus, the allantoic fluid was made 0.5 M in NaCl and 6% in polyethylene glycol (PEG), molecular weight 8000 daltons. This mixture was kept at 4°C overnight, the precipitate was then collected by centrifugation at 6000 rpm for 20 minutes in an RC2 Sorvall Refrigerated Centrifuge, using an SS34 rotor. The virus was purified on two discontinuous

(60, 40, 30, 15%) sucrose gradients and one continuous (60 to 15%) sucrose gradient run in a Beckman L3-50 ultracentrifuge using an SW28 rotor for two hours at 20,000 rpm. Three viral bands were collected after each run, one each from the 60-40% interface, the 30-40% interface, and the 15-30% interface. The sucrose was removed from the virus preparation by dialyzing against phosphate buffered saline (PBS) which was composed of 5.6g Na_2HPO_4, 2.7g KH_2PO_4, and 4.1g NaCl brought to one liter with water; pH was adjusted to 7.4. The virus was concentrated by dialyzing against PEG, molecular weight 20,000 daltons. The absorbance of the virus at 260 nm and 280 nm was measured and the protein concentration was determined with a 260/280 nomogram.

Myeloma Cell Line

The mouse myeloma cell line P3x63Ag8.653 (653 cells) was kindly provided by Dr. Don Graves from the University of Oklahoma Health Sciences Center. The cells were maintained in RPMI 1640 (K. C. Biological) containing 10% defined horse serum (HyClone), 1.0g/l $NaHCO_3$, 0.06g/l penicillin G, 0.10g/l streptomycin sulfate, and 0.30 g/l L-glutamine (SIGMA).

Immunization of Mice

Three BALB/c mice were injected intermuscularly with 0.1mg, UV-inactivated Sendai virus in Freund's complete

adjuvant. Three injections, one week apart were used and three days after the final boost, the fusion was performed.

Fusion Protocol

The fusion protocol used was adapted for this laboratory from the method used by Kohler and Milstein (45). The immunized mice were sacrificed, disinfected in Clorox solution, and the spleens removed asceptically. The spleens were then "washed" by placing them sequentially in three petri dishes containing a balanced salt solution (BSS). This solution was made as a 10X stock containing 80g NaCl, 4 g KCl, 3.5g $NaHCO_3$, 10g glucose, and 100 ml of 0.2% phenol red brought to a volume of one liter with water. The 10X stock was stored at room temperature with the addition of a small amount of chloroform. Before using, the solution was diluted and autoclaved. After the spleens were washed, the spleen cells were obtained by gently pressing the spleens through a nylon mesh placed over a petri dish containing serum-free RPMI 1640. These cells were pelleted at approximately 400 X g for seven minutes in a tabletop centrifuge. The pellet was resuspended in serum-free RPMI 1640, mixed with an equal number of 653 cells (pelleted under the same conditions as the spleen cells), and the pooled cells were centrifuged as before. The supernatant was discarded and to the pellet, 9ml (3ml/spleen) of 35% PEG (molecular weight 1000 daltons) in serum-free RPMI 1640, pH 7.8 was added.

The cells were gently resuspended in the 35% PEG and centrifuged at approximately 190 X g for two minutes. The cells were then allowed to incubate, undis turbed for six minutes at 30°C. SP medium, which consisted of 40% RPMI 1640, 40% conditioned RPMI 1640, 20% defined horse serum, and 0.05mg/ml gentamicin, was added to the cells to dilute the PEG. The cells were very gently resuspended and centrifuged at 190 X g for eight minutes. The pellet was resuspended in SP medium and the cells were divided into four T75 tissue culture flasks (Corning). The fluid level in each flask was brought to 40ml (cell concentration was approximately 1 X 10^8 cells/ml) and the flasks were incubated overnight at 37°C. The day after the fusion, the cells were pelleted at 400 X g for 10 minutes, resuspended in HAT medium, and seeded in 24 well tissue culture plates (Costar) at a concentration of 7 to 8 X 10^5 cells/ml. The HAT medium was made by adding 50 ul of aminopterin stock (3.51mg aminopterin per 10 ml 0.1N $NaHCO_3$) and 1 ml of HT stock (0.1361g hypoxanthine and 0.0388g thymidine in 100 ml of water warmed to 70 to 80°C) per 100ml of SP. The HT and aminopterin were passed through a 0.45um filter before adding to the SP medium.

The cells were kept in HAT medium for one week, followed by one week in HT medium (no aminopterin was added). After the second week, cells were fed with SP medium. Cells were fed twice per week by removing one-half the growth medium and replacing it with fresh

medium. Wells were screened microscopically for hybridoma growth and wells positive for growth were screened for the presence of anti-Sendai antibodies by enzyme linked immunosorbent assay (ELISA).

Enzyme Linked Immunosorbent Assay (ELISA)

Negative (pre-immune) and positive (immune) sera were obtained before or after immunization, respectively, by bleeding mice from the retro orbital plexus. The procedure for the ELISA was modified from Voller, et. al. (90). Ninety-six well Nunc plates (Gibco) were coated overnight at 4°C with either OSU T strain Sendai virus or the type strain from the American Type Culture Collection at a concentration of 85mg/ml in coating buffer. Coating buffer was composed of 1.59g Na_2CO_3, 2.93g $NaHCO_3$, and 0.2g NaN_3 made to one liter with water, pH 9.6. The plates were then washed three times with PBS, blocked with a 1 mg/ml solution of bovine serum albumin (BSA) in PBS for ten minutes, and washed again. Supernatants from the wells containing hybridomas, as well as positive and negative sera, were placed in the wells of the 96 well plate and incubated overnight at 4°C. After washing three times with PBS, sheep source anti-mouse antibody conjugated with alkaline phosphatase (SIGMA), diluted 1/1000 in PBS, was added to the wells and allowed to incubate for two hours at room temperature. The plate was again washed three times with PBS and the substrate,

p-nitrophenyl phosphate disodium (SIGMA), 1mg/ml in 0.2 M glycine buffer, pH 10.4, containing 0.001 M $MgCl_2$ and 0.001 M $ZnCl_2$, was added to the wells. Wells showing anti-Sendai activity turned from clear to yellow in color. Cells from wells containing positive supernatants were cloned in soft agar.

Soft Agar Cloning

The cloning medium used was SP plus Gibco amino acids with L-Glutamine and 5×10^{-2} M 2-mercaptoethanol. Five percent Difco agar in water was autoclaved and diluted in the cloning medium to a concentration of 0.5%. A cell suspension of 0.8 ml was added to 4.0 ml of cloning medium in a disposable petri dish. To this, 7.2 ml of the 0.5% agar was added and swirled, making the final concentration of agar 0.3%. Clones were visible to the eye approximately two weeks from plating and were removed with sterile pasteur pipettes. These clones were placed in fresh SP medium in 24 well tissue culture plates. When the cells had grown to sufficient numbers, the supernatants were retested for antibody production with the ELISA. Positive wells were recloned. All cells were cloned at least twice, some were cloned three times.

After the final cloning, it was necessary to determine to which protein each monoclonal antibody reacted with. Two assays were used to determine this, the hemagglutination inhibition assay and the Western Blot Assay.

Hemagglutination Inhibition Assay (HAI)

The hybridoma supernatants were tested by HAI to determine if the antibody present would block the hemagglutinin glycoprotein found on the surface of the virus. The assay was performed in disposable plastic trays (Linbro Chemical Company, Inc., New Haven, Conn.). A row of 12 wells was used for each hybridoma supernatant. Two hundred ul of PBS was added to each well, followed by the addition of 200 ul of supernatant to the first well of each row. A serial two-fold dilution of the supernatant was made in the remainder of the wells in each row except for the last two which were used as control wells. After the supernatant was diluted, 200 ul of virus solution containing four HA units per 200 ul was added to every well except the last well. The virus and antibody were incubated for 20 minutes at 37°C and then 200 ul of a 0.6% solution of chicken red blood cells in PBS was added to each well. When the control well containing only red blood cells and PBS showed a "button" and the control well containing virus plus red blood cells and PBS showed a "shield", the titer for each supernatant was determined. The titer was expressed as the inverse of the highest dilution of supernatant showing hemagglutination inhibition.

Western Blot Assay

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the viral proteins before transferring to nitrocellulose for the Western Blot Assay. A Bio-Rad Protean Slab Cell system was used and the gels were poured according to the Protean Slab Cell operating instructions. A 10% separating gel and a 3.5% stacking gel were used. The comb used was a one-well preparative comb. The separating gel had the following composition.

5.0 ml	1.5 M Tris-CL, pH 8.8
6.6 ml	30% acrylamide stock solution (Bio-Rad, 29.2 g acrylamide, 0.8g N^1N^1-Bis acrylamide made to 100 ml in water, filtered and stored at 4°C in the dark)
8.4 ml	Water
0.4 ml	10% SDS
10 ul	TEMED (Bio-Rad)
100 ul	10% ammonium persulfate

The stacking gel was made by mixing the following:

2.0 ml	0.5 M Tris-Cl, pH 6.8
1.1 ml	Acrylamide stock solution
6.76 ml	Water
0.1 ml	10% SDS
10 ul	TEMED
30 ul	10% ammonium persulfate

The virus sample for loading onto the gel was prepared by mixing 750 ul of the virus stock solution with 750 ul of Sample buffer (4.0 ml water; 1.0 ml 0.5 M Tris-CL, pH 6.8; 0.8 ml glycerol; 1.6 ml 10% SDS; 0.2 ml 2-mercaptoethanol; and 0.2 ml 0.05% (w/v) bromophenol blue). This mixture was heated to 100°C for four minutes and loaded onto the stacking gel.

The electrode buffer used consisted of 12 g Tris base, 57.6 g glycine, and 4 g SDS made to 4 liters with water, pH 8.3. Usually, two gels were run at one time. The current used was 18 milliamperes until the tracking dye had moved into the separating gel at which time the current was increased to 35 milliamperes. When the tracking dye reached the bottom of the glass plates, the gels were removed and placed into Transfer Buffer (12 g Tris base, 57.6 g glycine, 800 ml methanol, and water to make four liters, pH 8.3) for ten minutes.

Western Blot Assay

After soaking in Transfer Buffer, the gel and the nitrocellulose sheet were placed into the gel cassette according to the directions given in the 1983 Hoefer Scientific Instruments Catalog. The viral proteins were transferred for 45 minutes at a voltage of 100 volts. The nitrocellulose was removed from the cassette and strips were cut for both Coomassie Brilliant Blue Staining and India Ink staining. To stain the nitrocellulose with the Coomassie Blue, the strip was dipped into the stain (100 ml methanol, 20 ml acetic acid, 25 ml 1% solution of Coomassie Brilliant Blue, and water to make 200 ml) for approximately 45 to 60 seconds. The stain was then poured off and replaced by a destaining solution (250 ml methanol, 50 ml acetic acid and water to make 500 ml). Fresh destaining solution was added as needed. Both the

stain and destaining solution recipes were obtained from the 1983 Hoefer Scientific Instruments Catalog.

To stain the nitrocellulose strip with India Ink, a procedure by Hancock and Tsang was used (21). The strip was washed four times with PBS-Tween (PBS plus 0.5% Tween 20). Each wash lasted for ten minutes and was followed by a water rinse. The strip was then placed in the India Ink solution (1 ul of water soluble India Ink per ml of PBS-Tween) for 2 to 18 hours or until the protein bands were clearly visible. A destaining step was not required.

The remaining nitrocellulose was blocked with 5% nonfat dry milk in Tris Buffered Saline (TBS) (3.03 g Tris; 5.84 g NaCl; and water to make 500 ml, pH adjusted to 7.4 with HCl). The blocking step was for 30 minutes and was followed by three washes with TBS. The nitrocellulose was then cut into strips approximately 1/4 inch wide. Each strip was incubated in a different hybridoma supernatant for two hours. The strips were again washed three times in TBS and incubated an additional two hours in a Protein A - Peroxidase solution (SIGMA, 2 ug/ml TBS). The strips were washed as before and placed in the substrate which contained 1 ml 4-chloro-1-naphthol (SIGMA) in methanol (3 mg/ml) plus 5 ml TBS and 18 ul 3% hydrogen peroxide. When antibody to a particular viral protein was present in the supernatant, that particular protein band developed a purple color.

Liposome Assay

Antibody-loaded liposomes were made by the method of Darville et. al. (10) with some modifications. Soybean asolectin (20 mg/ml) was added to 5 ml of PBS containing a 1:50 dilution of goat anti-rabbit alkaline phosphatase conjugate (SIGMA). This mixture was incubated at 40°C until the asolectin had dissolved. The mixture was then vortexed at high speed for one minute, followed by a minute incubation at 40°C. These two steps were repeated for a total of five times. The liposomes were then centrifuged for 30 minutes at 10,000 rpm in a Sorvall RC-2 Refrigerated Centrifuge, using an SS34 rotor. The resulting pellet was washed three times in PBS. Each wash was for 20 minutes at 5000 rpm. After the final wash, the liposomes were resuspended in 3.0 ml of PBS and kept cold until needed.

Four dilutions (undiluted, 1/2, 1/4, and 1/8) of allantoic fluid and of allantoic fluid plus virus were prepared in serological test tubes. Each tube contained 0.2 ml. Tubes containing PBS and the third wash were also prepared. To each of these tubes, 0.2 ml of the liposomes were added and the tubes were incubated for 30 minutes at 37°C. After incubation, the tubes were centrifuged until a pellet containing the liposomes was observed. The supernatants were then tested for the presence of antibody by ELISA. A Nunc plate previously coated with rabbit serum was used. Supernatants from each tube were added to

the first well of a row and serially diluted with PBS in the remaining wells of each row. Also included was a row containing the supernatant from a tube of liposomes that had been heated to release trapped antibody. The supernatants were incubed in the wells for two hours. The plates were read the morning following the addition of the substrate with a Bio-Tek El-307 ELISA plate reader.

Antibody Blocking Assay

For the antibody blocking assay, monoclonal antibody ICB3-2C3-GC4 was used. Dilutions of the virus were the same as in the above liposome assay except the dilutions were made in the supernatant containing the antibody. The tubes were incubated for 30 minutes at 37°C, the liposomes were added and the assay was concluded as above. Control tubes containing cell supernatants but with no antibody were also included.

CHAPTER IV

RESULTS

Fusion

Five fusion attempts were made before one was successful and for each attempt made, the fusion protocol was slightly modified. Originally, the mice were not soaked in the Clorox solution after cervical dislocation nor were the whole spleens washed in BSS. These steps were added after the first fusion attempt showed gross bacterial contamination on the second day. The original growth medium used, RPMI 1640 supplemented with 20% Newborn Calf Serum did not support hybrid cell growth. Even after a change was made from Newborn to Fetal Calf Serum, the hybrid cells did not have the appearance of healthy myeloma cells although they did divide. When the conditioned medium was included to make the SP medium, the cells grew well even after a change, for economic reasons, from Fetal Calf Serum to Horse Serum was made.

At one point an "in vitro immunization" (65) was attempted. After the initial injection of antigen into the mice, instead of a booster injection, the spleen cells were harvested and put into T75 flasks. The cells were then "immunized" with the addition of 0.1 to 10 ug/ml of

antigen and the fusion was performed two to four days later. This particular fusion attempt succumbed to massive fungal contamination.

Amphotericin B has been used to help fight fungal contamination but not used routinely as the cells do not seem to grow as well when it is present. Instead, other methods were used to try to eliminate the opportunities for contamination, including allowing the plates to sit under the UV light for a few minutes before feeding the cells, using long pasteur pipettes when feeding to help eliminate the possibility of hands passing over open plates, and using guidelines restricting the use of the CO_2 incubator by non-authorized persons in the lab.

In addition, originally, 96-well tissue culture plates were used for plating out cells after the fusion. Fewer problems with contamination as well as greater ease in handling were encountered when the cells were plated into 24-well plates directly. The concentration of cells that has been used most successfully is 7 to 8 X 10^5 cell/ml. A higher concentration of cells seems to inhibit the growth of the hybridomas.

The fifth fusion attempt yielded several wells that contained antibodies to Sendai virus as tested by ELISA (Table I).

TABLE I

WELLS POSITIVE BY FIRST ELISA SCREEN				
1BB3	*3AC4	*4AB5	*4AB3	6AB3
1BC1	*3AA2	*4DC2	*5AB2	*6AA1
*1CB3	*3BD4	4CC3	5CC1	*6CA3
2AB3	*4AA5	*4DB3	6AB2	*7DB3

*wells that were cloned in soft agar

ELISA and Cloning

The OSU-T strain of Sendai virus proved to be a little difficult to work with in the ELISA. Although 1 to 10 ug/ml of protein is usually sufficient for coating the ELISA plates, better results were obtained using at least 85 ug/ml of this virus, making the assay "expensive" in terms of the man-hours needed to obtain the virus. Traditionally, PBS plus Tween 20 is used in the wash steps, however, with this strain of virus unlike the type strain, better results were obtained when PBS alone was used. In addition, the hybridoma supernatants were incubated in the ELISA plates overnight as opposed to the "normal" incubation period of two to three hours.

As wells that were positive for anti-Sendai antibody by ELISA were cloned, a method for identifying each clone became necessary. A code that allowed for the ability to trace the "family tree" for each clone at a glance was devised. For example, the clone 1CB3-2C6-DB4-CB2 originated from well B3 in the plate labeled 1C and was cloned three times with the numbers and letters 2C6, DB4, and CB2 identifying the plate and well numbers from which the cells were cloned the first, second, and third times, respectively. The clones 1CB3-2C3-FD1-GB2 and 1CB3-2C3-GC4-ED5 both were cloned two more times after originating from the same positive first clone well 2C3. Tables II, III, and IV list the wells that were positive for anti-Sendai antibody as tested by ELISA in the first,

second, and third clonings respectively. Figure 1 shows the same information diagramatically.

Interestingly, when the positive first clones were tested for antibody against the type strain of Sendai virus (chicken source), not all the hybridoma supernatants showed antibody activity to it even though antibody activity to the OSU-T strain was present (Table V).

Once the cells had been cloned, it was necessary to determine to which viral protein each monoclonal antibody reacted. Two assays, the Hemagglutination Inhibition Assay and the Western Blot Assay, were used.

Hemagglutination Inhibition Assay

Table VI lists several monoclonal antibodies that were tested for hemagglutination inhibition. Since the HN protein is responsible for hemagglutination of red blood cells, then antibody that inhibited hemagglutination should be directed against the HN glycoprotein. Table VI shows that all but one of the monoclonal antibodies tested (1CB3-2B6-DC4) inhibited hemagglutination and since that particular antibody was tested only once, it might have proved positive if retested, as were two others.

Western Blot Assay

Twenty-eight monoclonal antibodies were tested using the Western Blot Assay and all antibodies showed anti-HN activity while the control supernatants did not.

TABLE II

WELLS CONTAINING FIRST CLONES POSITIVE BY ELISA

(Positive First Clones)

Original Well #	1CB3			3AA2	3AC4	3BD4	4AA5
	2A4	*2C4	3C6	2A2	*2B3	*EA1	CA5
	*2A6	*2C5	*2D6	4B2		EA3	
	*2B4	2D4	*3A5				
	*2B6	*2D5	*3B3				
	*2C6	*3A6	*3B4				
	*2A5	3B5	*3D6				
	*2C3	*3C5	*2B5				

*wells that were cloned in soft agar

TABLE III

POSITIVE SECOND CLONES [a]

Positive First Clone #	1CB3-2C6	1CB3-2C3	1CB3-2B5	1CB3-2C5	1CB3-2C4	1CB3-2A5	1CB3-3D6	1CB3-2B4	1CB3-2D5	1CB3-2D6	1CB3-2B6	1CB3-3B4
	DD5	DC2	JA1	FD4	GD5	*DD4	DA6	*DA3	*DD1	FB1	JD5	*DB5
	KD3	*FD1		DC3	GA6		JA4		LA2		*FD3	
	*DB4	LC5		DA2			DC5		LC2		JB5	
		LD5		JB2			DD5				*DC4	
		GA3					KD5					
		GB3					KD4					
		GC3										
		*GC4										
		GD3										
		JD3										

*wells that were cloned in soft agar
[a]see text for explanation of code name

TABLE IV

POSITIVE THIRD CLONES [a]

Positive Second Clone #	1CB3-2C6-DB4	1CB3-2C3-FD1	1CB3-2C3-GC4	1CB3-2A5-DD4	1CB3-2B4-DA3	1CB3-2D5-DD1	1CB3-2B6-FD3	1CB3-2B6-DC4	1CB3-3B4-DB5
	DB2 CB1 CC1 CC2	GB2	ED5	CB5 CB6	EB5	GB5	EA3	AB1 AB2 AB3	CC3 CC4 CB3

[a]see text for explanation of code name

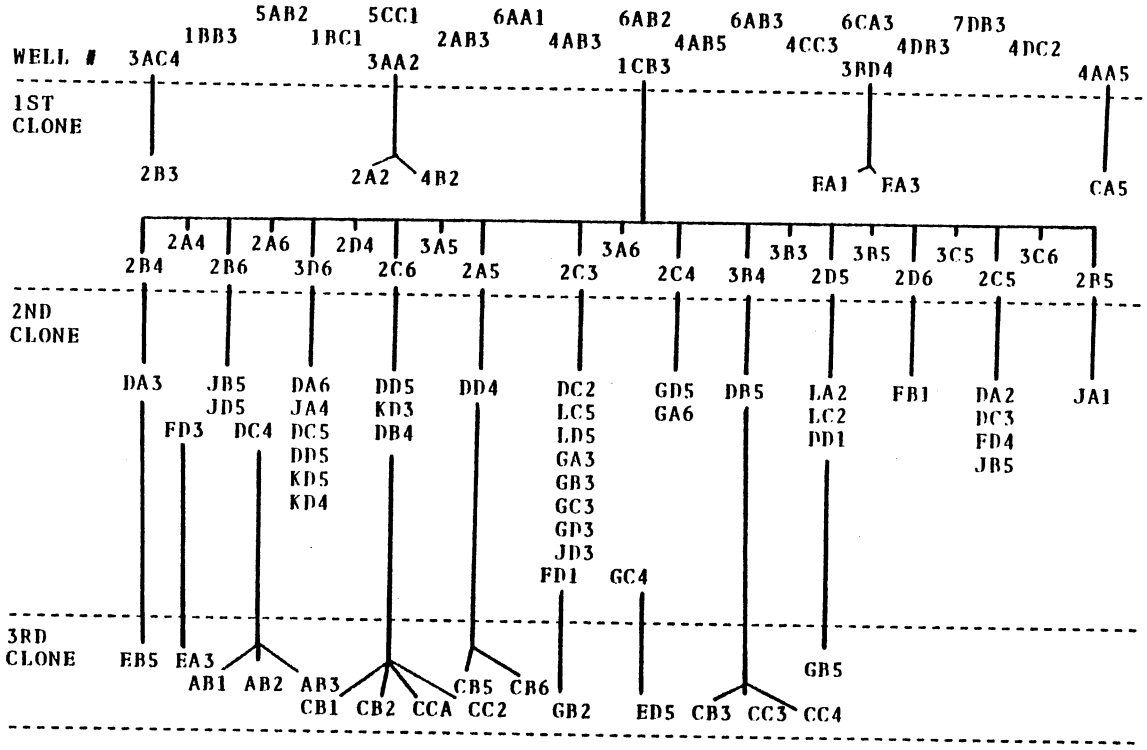

Figure 1. "Family Tree" for each monoclonal antibody

TABLE V

RESULTS OF ELISA: FIRST CLONES TESTED AGAINST BOTH OSU-T SENDAI (TURKEY) AND TYPE STRAIN (CHICKEN)

Well Number	First Clone	Anti-Turkey	Anti-Chicken
1CB3	2A4	++	-
	2A6	++	+
	2B4	++	+
	2B4	++	+
	2C6	+	+
	2A5	+	-
	2C3	+	-
	2C4	+	+
	2C5	+	-
	2D5	+	-
	2D4	+	-
	3A6	+	+
	3B5	+	+
	3C5	+	+
	3C6	+	+
	2D6	+	-
	3A5	++	-
	3B3	++	-
	3B4	++	-
	3C6	++	+
	2B5	+	-
3AA2	2A2	+	-
	4B2	+	-
3AC4	2B3	+	-
3BD4	EA1	ND	
	EA3	ND	
4AA5	CA5	ND	

++ strong
+ weaker than ++
- no antibody
ND not done

TABLE VI

HEMAGGLUTINATION INHIBITION ASSAY
Titer (HIU/ml)

Monoclonal antibody			Assay Number [a]				
			1	2	3	4	5
1CB3	2D5	FD4	0	80	40	20	10
1CB3	3D6	DC5	40	80	80	20	10
1CB3	2C3	GC3	40	40	40	20	10
1CB3	2D5	LA2	10	80	40	20	10
1CB3	3D6	DD5	40	80	80	20	
1CB3	2B6	JB5	10	80	80	20	10
1CB3	2C3	GA3	80	40	80	20	10
1CB3	2B4	DA3	0	80	40	20	10
1CB3	2C3	GD3		80	160	20	10
1CB3	2C3	GC4		160	160	20	10
1CB3	2B6	FD3		80	40	20	10
1CB3	2B6	DC4	0				
1CB3	2C3	GB3	80				
1CB3	3D6	DA6	40				
1CB3	2C5	DA2	80				
1CB3	2D5	DDA	80				
1CB3	3B4	DB5	160				
1CB3	2C6	DB4	80				
1CB3	2A5	DD4	160				

[a] Each assay was done at different times using supernatants from different cell cultures. No attempt to standardize the cell concentrations was done. The titers, then, for each antibody cannot be compared. However, the information obtained confirms the presence of antibody of the HN glycoprotein of Sendai virus.

Supernatant from cells (1CB3-2C5-DC4-AB3 and 1CB3-2C6-DC4-AB1) cloned from the one second clone (1CB3-2C6-DC4) which did not show hemagglutination inhibition did show anti-HN activity by the Western Blot Assay (Table VII).

Liposome Assay

Figure 2 shows results from a representative liposome assay. As the figure shows, the supernatant resulting from the centrifugation of the antibody loaded liposomes incubated with allantoic fluid containing Sendai virus shows the presence of a greater amount of released antibody than the supernatant from liposomes incubated with allantoic fluid with no virus (1:1 dilution). As the virus dose decreases, the amount of antibody released also decreases, indicating that the release of antibody from the liposomes is virus dependent. As the allantoic fluid is decreased, the amount of antibody released is slightly increased, indicating that perhaps the allantoic fluid may have a stabilizing effect on the liposomes. Several assays were done and all showed the same trend although comparing the different assays statistically is difficult since liposome preparations themselves and the amount of antibody incorporated varied.

Antibody Blocking Assay

Some preliminary studies were done using the monoclonal antibodies produced to the HN glycoprotein of

TABLE VII

RESULTS OF WESTERN BLOT ASSAY

Monoclonal Antibody				Protein Directed Against
1CB3	2B6	FD3		HN
1CB3	2C3	GO3		HN
1CB3	2C5	FD4		HN
1CB3	2D5	LA2		HN
1CB3	2B4	DA3		HN
ACB3	2C3	GA3		HN
1CB3	3D6	DC5		HN
1CB3	2C3	GC4		HN
1CB3	2B6	JB5		HN
1CB3	3D6	DD5		HN
1CB3	2C3	GD3		HN
1CB3	2C3	LC5		HN
1CB3	3B4	DB5	CB3	HN
1CB3	2B6	DC4	AB3	HN
1CB3	2C6	DB4	CB2	HN
1CB3	2B6	DC4	AB1	HN
ACB3	2C6	DB4	CB1	HN
1CB3	3B4	DB5	CC3	HN
1CB3	2D5	DD1	GB5	HN
1CB3	2C3	FD1	GB2	HN
1CB3	3B4	DB5	CC4	HN
1CB3	2B4	DA3	EB5	HN
1CB3	2C3	GC4	ED5	HN
1CB3	2A5	DD4	CB6	HN
1CB3	SB6	FD3	EA3	HN
1CB3	2A5	DD4	CB5	HN
1CB3	2C6	DB4	CC2	HN
1CB3	2B6	DB4	CC1	HN
Control				None

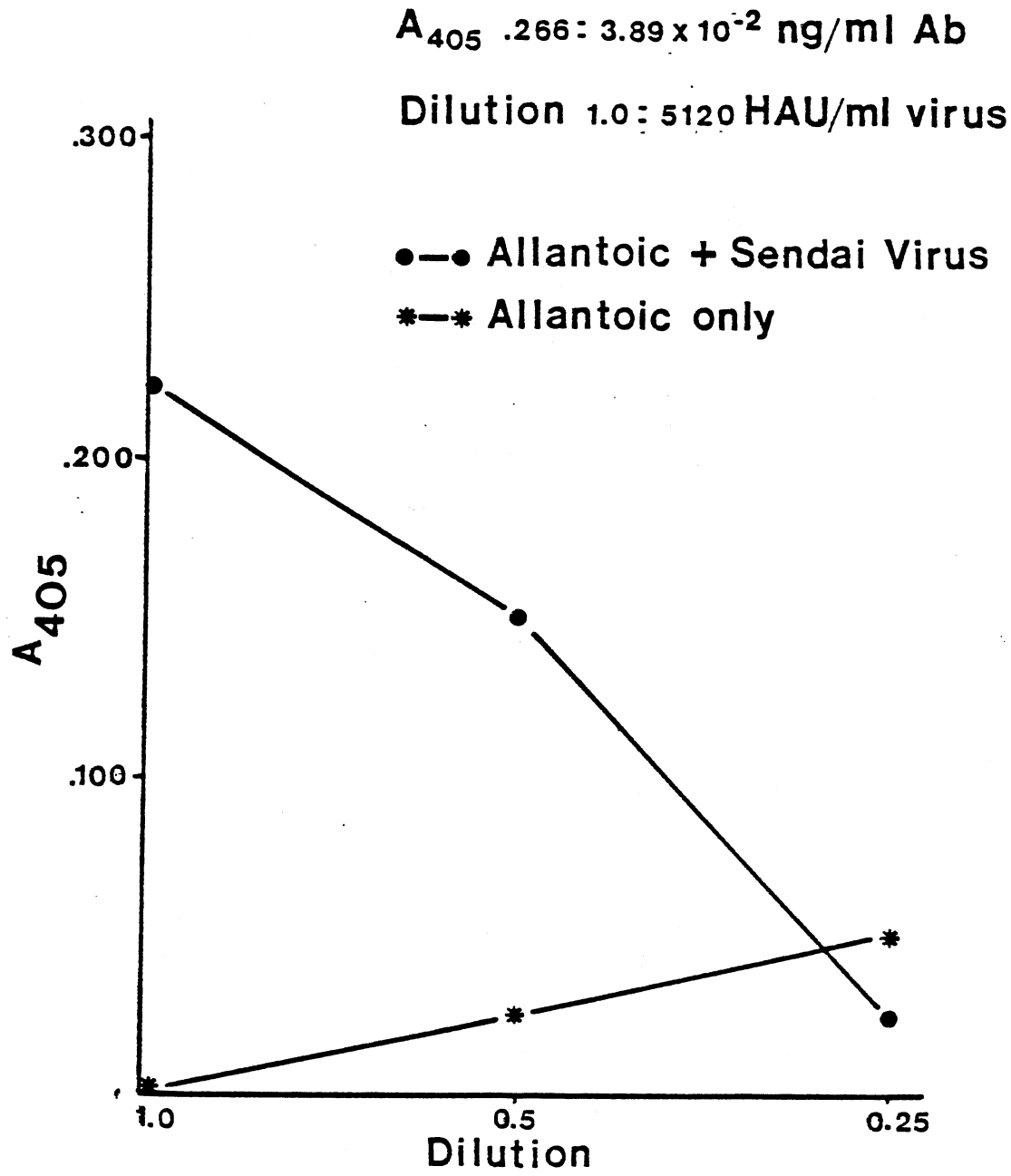

Figure 2. Results of Liposome Assay

Sendai virus. The virus was incubated with the antibody prior to the addition of the liposomes. No inhibition of the virus was seen under the conditions of the preliminary assay.

CHAPTER V

DISCUSSION

A monoclonal antibody bank to the OSU-T strain of Sendai virus has been started. While the techniques involved in the production of monoclonal antibodies have not been difficult, the trial and error methods necessary to develop successful procedures made the project lengthy, and even then, antibodies to all the viral proteins were not obtained. Despite the fact that all the monoclonal antibody producing cells obtained originated from the well, ICB3, and were directed against the HN glycoprotein, the evidence presented suggests that the antibodies are not identical. When isotyped, all the antibodies tested were IgG_1, but some showed kappa light chain secretion and others lambda light chain secretion. One antibody did not appear to inhibit hemagglutination even though by Western blot analysis it was directed against the HN glycoprotein. This possibility could exist since the HN glycoprotein probably possesses seven to eight antigenic determinant sites based on a molecular weight of 67,000. Not all of these sites would be expected to be involved in receptor activity. Lastly, some monoclonal antibodies reacted to both the OSU-T strain and the type strain of

Sendai virus when tested by ELISA, while others reacted only with the OSU-T strain.

In addition to the monoclonal antibodies produced, some information concerning the OSU-T strain of Sendai virus became apparent. When doing ELISAs, special steps were necessary when working with this strain that were not necessary when working with the type strain. The use of Tween 20 appeared to interfere with the assay when the OSU-T strain was used, suggesting that the binding of the virus to the ELISA plate was affected. Since the glycoproteins protrude from the surface of the virus particles, they are probably most involved in the binding of the virus to the plate and, thus, may be affected by the Tween 20. Since the Tween 20 did not interfere with ELISAs when the type strain was used, it is possible that the glycoproteins of the two strains differ in some way. This is supported by the fact that some of the monoclonal anti-HN antibodies that bound to the OSU-T strain did not bind to the type strain of Sendai virus. Perhaps, if a difference in the glycoproteins between the strains does exist, the entry of the virus, whether by fusion or some other method is affected.

Despite the fact that fusion seems to be widely accepted as the mode of entry by Sendai virus, many discrepancies concerning penetration by the virus exist in the literature. The fact that Sendai virus can cause fusion of cells seems to lead to the assumption that the

virus enters by fusing with the cell even though viropexis is also seen. It has even been suggested that those virus particles entering by phagocytosis are simply degraded in the cell (57). Even the permeability changes seen in cells infected with Sendai virus have been attributed to the "leakiness" due to the viral membrane that has been supposedly incorporated into the cell membrane rather than to the possibility that actual holes have been made in the cell membrane.

In these experiments, liposomes rather than cells were used as model membranes since the liposomes could be loaded with a substance that could be measured should that substance escape. In this case, labeled antibody that could be measured via the ELISA was chosen because of its ease in measurement and also because the antibody molecule is much larger than the pore size that has been suggested exists when Sendai virus fuses with a membrane (93). The data from these liposome assays indicate that the labeled antibody does indeed escape from the liposomes, suggesting that a hole of a larger size than one nm is created after infection with Sendai virus.

The fact that the liposomes used did not contain cholesterol could be used as a source of debate. As mentioned in Chapter II, some researchers believe that fusion required the presence of cholesterol (50) (73) and since these liposomes did not contain cholesterol, perhaps fusion <u>could not</u> occur. On the other hand, when Tsao and

Huang (89) measured leakage from liposomes they made, they stated that the presence of cholesterol suppressed the leakage from the liposomes. This would seem to support the idea that when cholesterol is not present, something other than fusion is occurring between the virus and the liposomes. However, Tsao and Huang speculated that the leakage they saw was due to penetration of the virus by fusion. Therefore, they have implied that the cholesterol suppressed fusion as measured by a reduction of liposome leakage. According to Haywood and Boyer (27), the presence of cholesterol is not necessary for Sendai virus to fuse with negatively-charged liposomes, so, according to their data, the OSU-T strain of Sendai virus could have fused with the liposomes.

A further source of debate could arise concerning the permeability change that accompanies Sendai virus fusion with membranes. It has been suggested that the pore established when fusion occurs is approximately one nm in diameter and that a molecular weight cut off of 1000 exists (93). The IgG molecule has a molecular weight around 150,000 and therefore could not escape from a pore only one nm in diameter.

In the preliminary antibody blocking assays, the monoclonal antibody to the HN glycoprotein did not appear to block the penetration of the virus. This could be due to several reasons. It is possible that the antibody did bind to an antigenic determinant of the HN in a way such

that the receptor binding capacity of the glycoprotein was not affected, or that the receptor binding portion was blocked, but F served as its own receptor (57) (75). This is not likely since the antibody used did block the HN protein in an HAI. A more probable explanation is that the anti-HN antibody did not block the action of the virus because the number of virus particles per antibody molecule present was too high. In this assay, 512 hemagglutinating units (HA) of virus were incubated with two hemagglutinating inhibition (HI) units. One HA unit is equal to 1×10^6 virus particles. Therefore, out of a total of 5.12×10^8 virus particles, only 8×10^6 were blocked, making the ratio of blocked to unblocked virus particles is 64:1. Obviously, a much higher concentration of antibody would be needed to be certain that the majority of virus particles were being blocked. An attempt to concentrate the monoclonal antibody supernatant by ammonium sulfate precipitation was made, but the resulting antibody preparation showed no hemagglutination inhibition activity. Perhaps another method of concentrating the antibody, such as affinity chromatography or the production of the antibodies in mouse ascites fluid can be attempted in the future. Once an antibody of high enough concentration is obtained, then these experiments could be repeated. Also, the isolation of a monoclonal anti-F is needed so that blocking assays using the two antibodies separately and together can be compared.

Based on work done by Seeman (85), Nishimura et. al. (66) have reported a technique in which they replaced the hemoglobin in erythrocytes with fluorescein isothiocyanate and rabbit IgG by a process they termed "gradual hemolysis." They used these erythrocyte ghosts as "syringes" and used Sendai virus to fuse the ghosts with target cells. This procedure might be beneficial in the study of Sendai virus penetration by providing a measurable substance (such as the labeled antibody used in the liposome assays) in a cell membrane. Antibody blocking assays could also be done.

While much work must still be done in the area concerning the penetration step of Sendai virus, a monoclonal antibody bank devised to aid in these studies, has been started. Included are several antibodies to the HN glycoprotein. Use of these antibodies has helped to raise the possibility that the glycoproteins of the OSU-T strain may differ from the glycoproteins of the type strain of Sendai virus. Liposome assays that indicate a hole large enough for an antibody molecule to escape is produced upon infections with Sendai virus and preliminary anti-Sendai blocking assays have been done, as well as suggestions made for further investigations in this area of controversy.

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