CHARACTERIZATION OF A NEW STRAIN OF SENDAI VIRUS AND ITS ABILITY TO INDUCE AUTOIMMUNITY IN TURKEYS

By

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CHAPTER I

INTRODUCTION

The division of viruses known as paramyoxviruses is a well-defined family. These viruses are large and contain negative-stranded ribonulcleic acid as their genetic material. Paramyxoviruses contain a lipid envelope which is obtained from the host cell plasma membrane. The host plasma membrane is modified with viral-specific proteins before the virus particles are released. It is the effect of the modification of normal plasma membrane that will be investigated here.

The mechanisms of implantation of the viral-specific proteins has been elucidated by other investigators; therefore, this process will not be elaborated upon. Instead, the majority of this study will be devoted to how the host animal "sees" this modification of its cells' plasma membranes immunologically and what effect the viral-specific protein have on the infectivity of the progeny virus. How these two areas relate to Sendai virus is of particular interest in this study.

Sendai virus was orginally isolated during an epidemic of fatal pneumonitis in children in Sendai, Japan, in 1952 (20). Kuroya observed that Sendai virus would replicate in

embryonated chicken eggs, thus providing an easily available source for the production of virus. The ability of Sendai virus to agglutinate chicken red blood cells was also observed at the same time. In 1955 Fukai and Suzuki (9) demonstrated that Sendai virus exhibited hemolytic activity. The host range of Sendai virus has been found to include human, hamsters, mice, swine, guinea pigs and others (20, 26).

The physical structure of Sendai virus is typical of the paramyxo group. The virion was shown to be spherical to pleomorphic when negatively stained and examined with the electron microscope (15, 2). The diameter of the virion was shown by Kosaka et al. (16) to range from 120 nm to 300 nm. As mentioned earlier, Sendai virus contains an envelope which is a modified host-cell membrane containing projections of viral glycoproteins. The hemagglutinating and neuraminidase activity of Sendai virus has been attributed to these glycoprotein projections (15). The viral envelope also contains an inner protein layer which is not found in the host cell membrane and is referred to as the matrix protein (16). The matrix protein surrounds the nucleocapsid. The single strand of negative-sense ribonucleic acid (RNA) is located within the nucleocapsid, which is composed of a single-stranded, left-hand helix containing 11 to 13 subunits in each turn of the helix (7). Lamb and Mahy (21) reported that depending upon the host system, Sendai virus has 8 to 9 structural proteins ranging in molecular weight from

3.47×10^4 to 1.45×10^5 daltons. However, Mountcastle et al. (27) observed Sendai virus to contain six and possibly seven structural proteins ranging in molecular weight from 3.8×10^4 to 6.9×10^4 daltons.

The biological functions of the major proteins, however, have been agreed upon. According to Lamb et al. (21), the proteins are as follows: a nuclear protein (NP), a glycosylated protein associated with hemagglutinating and neuraminidase activity (HN), a glycosylated protein associated with cell fusion and hemolysis activity (F), a membrane matrix protein (M) and two internal proteins involved in RNA transcriptase activity. The F glycoprotein is present either as an inactive precursor F_o or as a cleaved and active form consisting of two disulfide-linked chains, F_1 and F_2. The cleavage of the F protein has been reported to depend on the host system used to grow the virus (31, 19).

Parameters of Biological Activity Affected by F_o Cleavage

The biological activity of Sendai virus grown in the chorioallantoic cavity of fertile chicken eggs has been shown to be different from the biological activity of Sendai virus obtained from the culture fluids of cells infected with egg Sendai. Table I lists these differences. Ishida and Homma (19) were the first to observe that Sendai virus (CCS) obtained from the fluids of cultured cells infected by

egg Sendai virus, was not infectious when added to freshly cultured cells. However, the CCS was infectious when injected into fertile chicken eggs, and the resulting virus reverted to the properties shown by egg Sendai. The theory was then put forward by Ishida, Homma and others (18, 24, 19) that the variation in host range for a given system was not heritable, and the phenomenon was described as a host-induced modification.

The possiblity of a defect in the absorption mechanism of CCS was eliminated as a cause for this phenomenon when it was observed that CCS absorbed to cultured cells at $4^{o}C$ at approximately the same rate as egg source Sendai. The CCS, however, was eluted from the cultured cells when the temperature was raised to $37^{o}C$, whereas the egg source Sendai proceeded to penetrate the cultured cells (18).

As shown in Table I, the ability of the CCS virus to lyse red blood cells and fuse cells was also lost with the infectivity (18, 14). These findings revealed two important parameters: there exists a close relationship between hemolysins and cell-fusing activities, and they have an important role in the process of Sendai virus penetration of cells (18).

The inability of CCS to infect cultured cells prevents egg Sendai from acting as infectious centers when plated on a monolayer of cultured cells. This lack of infectivity thus prevents the formation of plaques. However, Shibuta (32) revealed that when CCS was plated on monolayer cultured

TABLE I

GENERAL CHARACTERISTICS OF SENDAI VIRUS

Characteristic	Egg Sendai	Cultured Cell Sendai (L)
Hemagglutinin	+	+
Neuraminidase	+	+
Infectivity for eggs	+	±[a]
Infectivity for cultured cells	+	-
Hemolyzing activity	+	-
Cell-fusing activity	+	-
Plaque formation on cultured cells	-	-

[a] The infectivity of L Sendai for eggs was always lower than that of egg Sendai. The ratio of the infectivity to the hemagglutinating titer was $10^{4.0-4.5}$ for L Sendai and $10^{6.0-6.3}$ for egg Sendai.

cells with the presence of trypsin in the overlay agar, plaques appear. At the same time it was observed that the hemolysin and cell-fusing activities also were regained when CCS was incubated with trypsin (12). The activation of the non-active CCS to the active form now has been shown to be a result of the direct action of trypsin on a virion-coded glycoprotein (13, 31, 28).

Polyacrylamide gel electrophoresis (PAGE) of egg Sendai and CCS revealed a relationship between the structural proteins responsible for biological activity and the direct effects of trypsin. The biologically active egg Sendai, as well as the CCS which had been treated with trypsin, was devoid of the glycoprotein F but contained large amounts of the F_1 and a small amount of F_2 glycoproteins. The biologically inactive CCS, in comparision, contained only a large amount of the F protein (13, 31, 33). A precursor-product relationship of F and the small glycoproteins F_1 and F_2 was suggested by the fact that the increase in F_1 and the appearance of F_2 occurred simultaneously with the disappearance of F, and that the sum of the molecular weights of F_1 and F_2 roughly balanced the molecular weight of F (19).

Modification of biological and physical properties of animal viruses by host cells has been reported by many workers. In contrast to phenotypic variations attributed to mutation, such modifications are brought about regularly in certain hosts during the course of a single growth cycle of

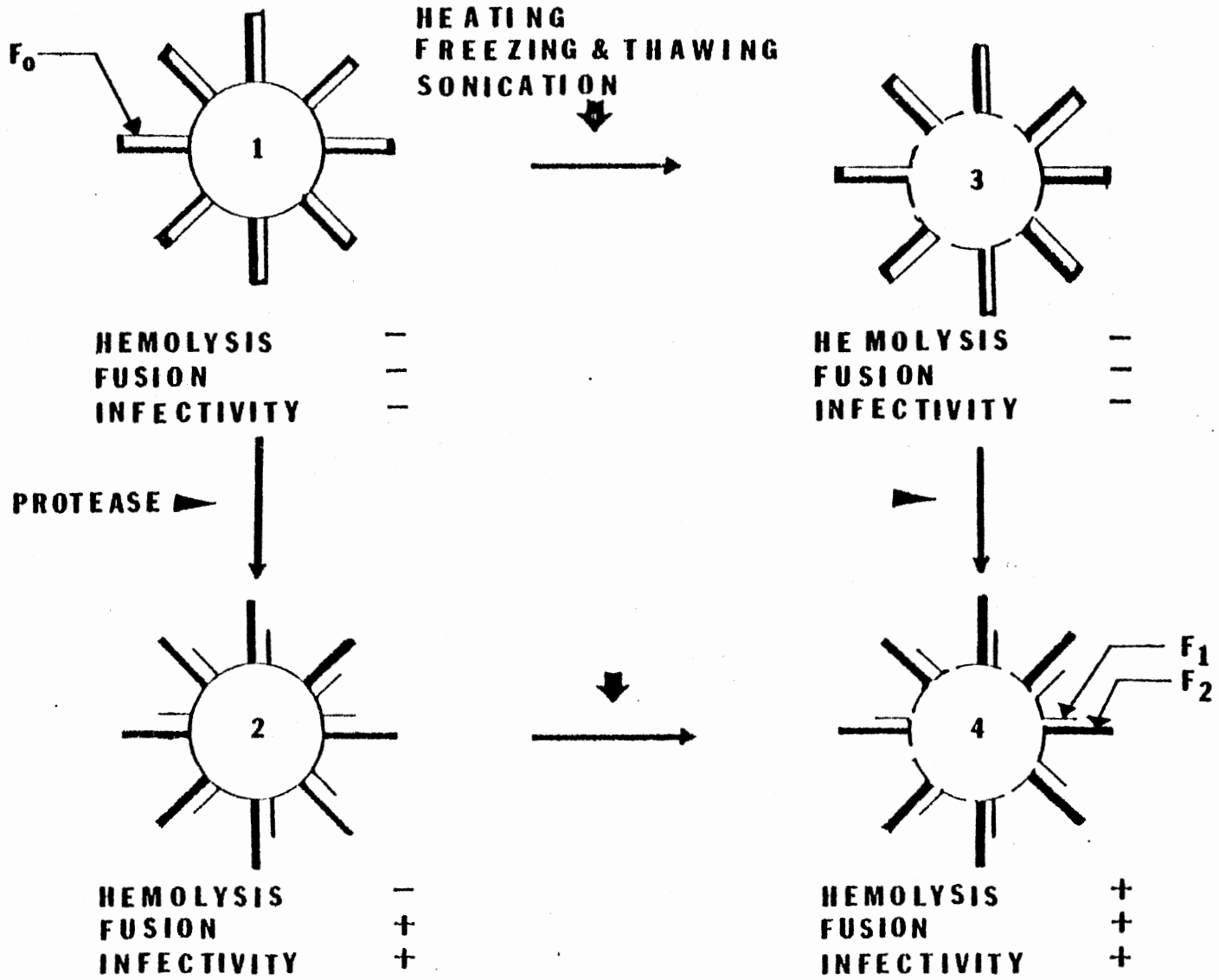
the virus. The modification is not heritable, and the modified virus can be converted to the original form after a single passage in the original host. Whatever the mechanism, since this type of change is apparently controlled by the host cell, the phenomenon has been called host-induced or host-controlled modification; it is commonly observed in enveloped RNA viruses, and frequently observed in paramyxoviruses (19).

The modification of Sendai virus is summarized in Figure 1. Physical treatment, e.g., incubation at 36°C, freezing and thawing, or ultrasonication, converts the virons from type 1 to type 3, or from type 3 to type 4 by causing alteration of the envelopes (dashed line). When the virions have F_1 and F_2 glycoproteins, hemolysis may occur following envelope fusion (type 4). Protease treatment converts the virion from type 1 to type 2 or from type 3 to type 4 by cleavage of F to F_1 and F_2 glycoproteins and results in envelope fusion (types 2 and 4). Hemolysis may occur when the virion has an altered envelope (type 4) (19).

Implication of Paramyxoviruses in Autoimmune Diseases

In recent years, the paramyxovirus family has been suspected of being the etiological agent, either directly or indirectly, for chronic diseases of the central nervous

Figure 1. Model of Sendai virus modification (18).


HEATING
FREEZING & THAWING
SONICATION
F_o
1
3
HEMOLYSIS −
FUSION −
INFECTIVITY −
HEMOLYSIS −
FUSION −
INFECTIVITY −
PROTEASE
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4
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F_2
HEMOLYSIS −
FUSION +
INFECTIVITY +
HEMOLYSIS +
FUSION +
INFECTIVITY +

system such as mutiple sclerosis (MS) and subacute sclerosing panencephalitis (SSPE). How the viruses act is not known exactly; however, three main theories have been suggested by various investigators. These theories, all of which have some supporting evidence, are: the infecting virus is always present and produces slow cytopathic effects; the virus is latent in the infected cell but does produce viral proteins which the individual's immune system attacks at the cost of "killing" the infected cell; and the virus elicits an autoimmune response by forming a neoantigen composed of virus proteins and normal cell proteins which the host immune system attacks.

The slow virus theory of a persistent chronic virus infection became a credible position when Carp et al. (1) isolated a small virus-like agent found in association with multiple sclerosis material. The Carp agent was found in 90% of the MS patients used in the experiment. The agent was found to be between 25 and 50 nm in diameter by filtration, and replicated in baby hamster in high titers. Carp et al. also reported the presence of the agent in serum, cerebral spinal fluid (CSF), spleen, kidney and lymph nodes. The agent was not observed in any of the control samples. So far as cytopathic effects are concerned, the highly significant attribute of this agent is that it has been detected so far only by its effects on haemopoietic tissue, or on cells which are already transformed; thus the agent has been observed only in cells carrying another virus genome. It

may well, therefore, reactivate a latent virus which then participates in the observed pathogenesis (8).

Paramyxoviruses or paramyxovirus-like particles have also been observed in tissue collected from MS plaques; however, few attempts at isolation have been successful (35). Paramyxoviruses, as well as other viruses, are known to be latent in brains of monkeys and man without producing cytopathic changes (30).

The fact that other slow virus diseases also exist adds to the credibility of a slow virus etiology for MS. Kuru and Creutzfeld-Jakob disease are two examples of brain-related progressive degenerative conditions which are associated with virus like agents (10). Mouse Hepatitus Virus Encephalmyelitis (MHVE) and Progressive Multifocal Leukoencephalopathy (PML) are examples of diseases in which viruses are directly responsible for demyelination (23).

The theory that MS may be related to a latent virus infection in which actual progeny virus is not produced but viral proteins are has been based chiefly on studies of Subacute Sclerosing Panencephalitis (SSPE). In contrast to demyelination in PML and MHV encephalomyelitis, which is caused by virus-induced lysis of oligodendrocytes without demonstrable participation of immunologic reaction, the pathogenesis of SSPE includes interaction of virus and virus-infected cells with host immune responses (23). After many unsuccessful attempts to recover a virus from SSPE samples or to transmit the disease, attention focused on measles

virus when electron micrographs revealed paramyxovirus-like nucleocapsids in the nuclei of affected brain cells (34). At approximately the same time, measles virus antigens were demonstrated in tissue sections, and evidence for local production of anti-measles virus antibodies was obtained. Payne et al. (29) were able, by cocultivation techniques, to recover several variants of measles virus from brain cells despite the presence of antibodies that are bound to infected cells. Smooth nucleocapsids indistinguishable from those observed in wild-type measles infected cells have been observed beneath the plasma membrane in oligodendrocytes; however, budding of complete virus never seems to occur and the nucleocapsid is distributed in both cells upon mitosis (23).

The assertion that MS and SSPE are closely related is based upon several findings. The observation of paramyxovirus-like nucleocapsids beneath the plasma membrane of cells obtained in a biopsy of MS plaques suggests the virus is present (35). Hyridization studies of virus nucleotide sequences done <u>in situ</u> have shown measles virus to be present in material obtained from 1 of 4 MS patients. This procedure was able to detect the virus genome present in infected cells which did not demonstrate viral antigens on the cell surface (11). The fact that most MS patients maintain an abnormally elevated titer of antibodies against measles virus in their serum and in their cerebral spinal fluid would also indicate a persistent measles virus

infection.

The possibility that neoantigens, formed by a virus in connection with normal host proteins, are responsible for an autoimmune basis for MS is demonstrated by several recent investigations. Eaton (3) demonstrated that absorption of paramyxoviruses, including Sendai, to tumor cells produces neoantigens that are immunogenic in syngeneic mice, thus imparting resistance, whereas similar preparations from uninfected tumor cells are not immunogenic. Syngeneic mice produce antibodies cytotoxic for uninfected tumor cells and cell mediated immunity. Also, complement-fixing antibodies were detected which were reactive with the membranes and several other tumor cell fractions. Antibodies were not stimulated by similar preparations from uninfected tumors. In addition to the anti-tumor immunity, autoantibodies against syngeneic spleen, as well as other tissues, were demonstrated. With continued immunization, antibodies to deoxyribonucleoprotein and heterophile reagins also appeared, and some of the mice died with a wasting disease characterized by a loss of lymphoid tissue and scarred, atrophied kidneys. These observations on the effects of absorbed virus indicte that modification of cell membrane antigens is a biochemical reaction that can occur in the absence of viral replication, causing syngeneic or "self" antigens to appear "foreign" to the humoral and cellular response system (4). Finberg et al. (6) demonstrated that infection of mice, after they had been environmentally primed by Sendai

virus, produced cytolytic activity against allogenic antigens. He observed that the killer cells were T lymphocytes.

This investigation was concerned with two areas of Sendai virus biology. First the characterization of a new strain of Sendai virus known as OSU-T relating to its morphology, protein composition, and plaquing ability is reported in order to determine any unique properties of the viurs. Additionally, the effects of modification of the normal plasma membrane of turkey embryo brain (TEB) cells by Sendai virus was studied. The altered TEB membranes were tested for their ability to induce an autoimmune response in healthy turkeys.

The information obtained from this study will be important in determining if neoantigen formation by Sendai virus is a probable cause of chronic diseases of the central nervous system.

CHAPTER II

MATERIALS AND METHODS

Water

All water used in the following methods was obtained from a nanopure water system (Barnstead) unless otherwise indicated. The water system was maintained in such a way as to deliver only water with a resistance of approximately 18 n ohms.

Growth Media 1X, 2X, BME or MEM

The base for growth media was either Basal Medium Eagle (BME) or Minimal Essential Medium (MEM) (KC Biological, Kansas City, Kansas). Both bases contained L-glutamine, but did not contain $NaHCO_3$. Both bases were used in a concentration of 10.3 g/l. To the base medium 9.53 g of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer was added, along with streptomycin sulfate (100 mg/l), penicillin G (60 mg/l), and either 5% v/v or 10% v/v calf serum. Tryptose phosphate broth was added (5% v/v) to BME. The growth media were adjusted to a pH of 7.2 with 5.0 N NaOH, filter sterilized, and stored frozen at -20°C until needed.

Growth media 2X (BME and MEM) contained the same components as growth media 1X; however, the total volume was adjusted to 500 ml rather than 1 liter.

Balanced Salt Solution Without Ca^{++} and Mg^{++} (BSS) 10X

BSS-10X was made by combining 80 g NaCl, 4 g KCl, 3.5 g $NaHCO_3$, 10 g glucose and 100 ml of 0.2% phenol red. The total volume was brought to 1 liter with water. The 10X stock solution was stored at room temperature with the addition of a small amount of chloroform. The stock was diluted to a 1X solution and autoclaved at 121°C, 15 psi for 15 min before being used as a wash for either whole brain tissue or monolayer tissue cultures.

Trypsin-EDTA Solution

Trypsin-EDTA (Disodium ethylene diaminetetracetic acid) solution, used to disrupt tissue for cell culture, was prepared as follows: Pure powdered trypsin (0.02% w/v) was dissolved in 1X BSS which contained 0.02% w/v EDTA. The solution was filter sterilized and stored at -20°C. Concentrated solutions (10X) of trypsin-EDTA were obtained from Gibco, Grand Island, N.Y.

Phosphate Buffered Saline (PBS) 10X

PBS was used as a diluent and to wash cells. The stock solution of PBS 10X was prepared by mixing Na_2HPO_4 (55.9 g), KH_2PO_4 (27 g) and NaCl (41 g) in 1 liter of water. The stock solution was diluted 1:10 in water and the pH adjusted to 7.2 before use.

Alsever's Solution

Alsever's solution was used in collection and storage of chicken, turkey and sheep blood used in some assay systems. This solution was prepared by mixing glucose (2.05 g), sodium citrate (0.8 g), NaCl (0.42 g) citric acid (0.02 g) and 100 ml of water. The solution was stored at $4^{o}C$. Whole blood was diluted 1:1 with Alsever's solution.

Red Blood Cell (RBC) Suspensions

Chicken and sheep RBC's were used in various assays. Standard solutions were made by collecting whole blood in Alsever's solution, centrifuging at 1700 rpm in an International Clinical Centrifuge Model CL for 5 min, and washing the pellet 3 times in PBS. The final pellet was resuspended to a concentration of 0.6% packed cells/ml for chicken RBCs and 2% packed cells/vol for sheep RBCs.

Tris Swelling Buffer

Tris (hydroxymethylaminomethane) swelling buffer was used to osmotically rupture tissue culture cells for plasma membrane isolation. This solution was prepared by combining 0.121 g of Tris/100 ml water and adjusting the pH to 7.6 with 12 N HCl to yield 0.01 M Tris-HCl solution.

Overlay Medium

Overlay medium was used for plaque assays of Sendai virus infected turkey cells. Special agar-Noble (Difco) (1.5%, 2X) was sterilized by autoclaving. The melted 2X agar was cooled to 45°C and added to an equal volume of 2X growth medium (MEM or BME) (45°C).

Neutral Red Agar Overlay

Plaques caused by Sendai virus were developed by addition of neutral red agar. Neutral red agar overlay had the same composition as the overlay medium except that neutral red dye was added (1:10,000) to the 2X agar prior to autoclaving.

Solutions for Slab Gel Electrophoresis

The following solutions were used for polyacrylamide slab gel electrophoresis. A standard solution of 30% acrylamide plus 0.8% N,N'methylene bisacrylamide was dissolved in water and filtered through Whatman No. 1 paper to remove particulate

matter. This solution was stored at 4°C in the dark. Sodium dodecyl sulfate (SDS) (10%) was used as a stock solution.

Upper Tris buffer used for buffering the stacking gel and well-forming gel was composed of: Tris base (6.06 g), SDS stock solution (4 ml), water (80 ml), and the pH was adjusted to 6.8 with 12 N HCl and the final volume of the solution was adjusted to 100 ml with water.

Lower Tris buffer used for buffering the running gel was composed of: Tris base (18.17 g), SDS stock solution (4 ml), and water (80 ml). The pH was adjusted to 8.8 with 12 N HCl, and the final volume of solution was then adjusted to 100 ml with water.

Tris-glycine reservoir buffer was composed of Tris base (12.1 g), glycine (57.6 g), SDS (4.0 g), and water to bring the final volume to 4 liters. The final pH was between 8.2 and 8.4.

Ammonium persulfate (10%) was used as the initiator of polymerization. The solution was made fresh each time.

Polyacrylamide gels were fixed in a solution composed of isopropanol (25%), glacial acetic acid (10%) and water (65%). The gels were stained in the same solution with the addition of Coomassie Brilliant Blue (0.05%).

The gels were destained in a solution of glacial acetic acid (10%) and water (90%).

Virus Stock

Two strains of Sendai virus were used in this investigation. The strain which has been designated as OSU-T Sendai was used in all experiments, except when a reference as to how this strain has changed from the originally isolated Sendai virus was needed. For the purpose of comparison, the original strain of Sendai virus was acquired from the American Type Culture Collection and was designated VR-105.

OSU-T Sendai virus was isolated by Dr. Mark R. Sanborn, Department of Microbiology, Oklahoma State University. The exact passage history of the strain is unknown; however, the strain was isolated from a chicken strain provided by Dr. David Kingsbury at St. Jude Research Center in Memphis, Tenn. The OSU-T strain has been propagated in turkey eggs at Oklahoma State University for 5 years. To verify that the virus was indeed Sendai virus, 0.2 ml of virus stock was neutralized in an HAI titration with Sendai virus typing antiserum provided by the National Institute of Allergy and Infectious Diseases, Bethesda, Md. OSU-T Sendai was also examined with the electron microscope to verify size and morphology.

OSU-T Sendai virus was propagated in 14 day-old embryonated turkey eggs. Turkey eggs were candled to determine fertility, then incubated under ultraviolet light for 20 min. A small hole was placed in the blunt end of the eggs, and a sterile stock solution of infectious allantoic fluid was injected (02 ml) with the diluted virus solution

(4 HAU/0.2 ml). The eggs were incubated at 31°C for 48 hr, and refrigerated at 4°C for 2 hr before the allantoic fluid was harvested. The crude allantoic fluid was stored at -20°C. Purification of virus from allantoic fluid was initiated when 1 liter of allantoic fluid was accumulated. Allantoic fluid was clarified by low speed centrifugation at 3000 x g (4°C, 30 min). The supernatant was then layered onto a 10 ml sucrose cushion (60%) in a nitrocelluose-ultracentrifuge tube and centrifuged at 70,000 x g (4°C, 2 hr). The opaque layer directly above the sucrose cushion was extracted with a syringe and curved 16 gauge needle. The virus solution was then concentrated to dryness by dialyzing against polyethylene glycol (20%; 4°C), molecular weight 6000, in dialysis tubing with a molecular weight exclusion of 12,000 daltons. The virus was resuspended in a minimal amount of PBS and layered onto a discontinuous sucrose gradient composed of (top to bottom): 10 ml, 15%; 10 ml, 30%; 10 ml 40%; and 5 ml, 60% sucrose. The gradients were centrifuged at 70,000 x g, 4°C for 2 hrs. The opaque band was again extracted with a syringe and curved 16 gauge needle. The virus was concentrated again by dialysis and resuspended in a minimal volume of PBS. The virus fraction was then layered onto a continuous sucrose gradient (24 - 58%) formed by a Beckman Linear Gradient Former and centrifuged at 70,000 x g, 4°C, for 2 hrs. The opaque bands were again collected as mentioned above and frozen.

Strain Sendai/52 (ATCC VR-105) from the American Type Culture Collection was propagated in 10 day-old fertile chicken eggs and purified in the same manner as OSU-T Sendai virus.

Source of Turkey and Chicken Eggs

Turkey eggs used for virus propagation and embryonic cell cultures were generously donated by the Oklahoma State University Animal Science Department, Stillwater, Okla., and the Nicholas Turkey Breeding Farm, Inc., Sonoma, California. Eggs were also purchased from the Armour Hatcheries in Brownwood, Texas.

Chicken eggs used for virus propagation and embryonic cell culture were also donated by the Oklahoma State University Animal Science Department, Stillwater.

Injection and Observation of Turkeys

The Animal Science Department at Oklahoma State University supplied and maintained the forty White Hollin turkeys used in part of this investigation. The turkeys were divided into two groups of twenty birds without being sexed. Two weeks after birth, the birds were weighed, banded and observed before experimentation began. Each of the twenty birds in the control group were injected intramuscularly with 1 ml of a suspension of normal TEB membranes which contained 1 mg

of protein. Each of the birds within the experimental group received an injection of 1 ml of a suspension of TEB membranes modified by Sendai virus which contained 1 mg of protein. All birds were examined for neurological disturbances by testing the ability of the bird to right itself after being placed on its back. Serum samples were not obtained after the first injection because the birds were physically too small. Each subsequent week the birds were injected with 1 mg of the appropriate TEB membrane suspension, normal membranes for control birds, modified membranes for the experimental birds. Serum samples, weights and righting response were also obtained each week. Injections were continued for a total of twenty-six weeks. At the end of the twenty-sixth week the birds were weighed and the righting response tested one last time. The birds were then sacrificed and as much serum as possible was obtained from each bird. The brains of the birds were also extracted and preserved in neutral buffered formalin. An attempt was made to draw cerebral spinal fluid also.

Turkey Embryo Brain Cells
(TEB Cells)

TEB cells used for injection, lymphocyte adherence test, complement fixation and fluorescent antibody tests were cultured as follows: Fourteen to eighteen day-old-fertile turkey eggs were soaked with a solution of ispropanol (100%) and placed under ultraviolet light in a

Bioquest Biological Cabinet (Laminar airflow sterile hood). The healthy embryos were removed aseptically using curved forceps and placed in sterile disposable plastic petri plates. The embryo's brains and upper portion of the spinal cord were gently removed and placed in another sterile petri plate which contained sterile BSS (30 ml). When a suitable number of brains had been collected (10-30), they were cut into 1 mm pieces using two sterile scalpels. These pieces were collected and transferred to a sterile 125 ml trypsinizing flask, using a sterile needle and syringe (10 guage; 30 ml). Sterile trypsin-EDTA (50 ml) was then added to the flask, and the tissue solution was stirred gently until it became opaque. The large pieces of tissue were then allowed to settle out, and the supernatant was removed and poured into a sterile 15 ml screw-capped test tube. Cold trypsin-EDTA (50 ml) was again added to the remaining large pieces of tissue and the stirring was resumed. At the same time, tubes containing the supernatants (cell suspensions) were centrifuged (500 g, 5 min) to pellet the cells. Upon completion of centrifugation, the trypsin-EDTA supernatant was aseptically poured off and replaced by a minimal amount of sterile MEM (10% calf serum). The cells were then resuspended by vortexing and filtered through sterile gauze into a sterile 1 liter flask. This procedure was repeated until no large pieces of tissue remained in the trypsinizing flask. The concentration of cells in the MEM cell suspension was then determined by direct count using a

hemocytometer. The cell concentration was adjusted to 2.0 x 10^6 cells/ml. Various tissue culture flasks received the following amounts of cell suspension: surface area glass and Falcon plastic roller bottles received 100 ml, 75 cm^2 Lux and Corning plastic flasks received 20 ml, and 100 ml medicine bottles received 15 ml.

TEB were cultured at 37°C in MEM (10%) growth medium. The cells were fed once or twice with MEM (5%) before harvesting.

Turkey Embryo Fibroblast (TEF) Cells

TEF cells were used to determine the plaquing ability of various strains of Sendai virus. Twelve to sixteen-day old fertile turkey eggs were soaked with isopropanol (100%) and exposed to ultraviolet light in a Bioquest Biological Cabinet. The healthy embryos were aseptically removed from the eggs with curved forceps and placed in a sterile plastic petri dish. The head and viscera were removed and the remaining body was transferred to a sterile petri dish which contained BSS (30 ml). When a suitable number of embryos was collected (2-6), the bodies were placed in the tube of a sterile 30 cc syringe and expressed into a 125 ml trypsinizing flask by exerting pressure on the plunger. Trypsin-EDTA solution was then added to the macerated tissue. The procedure from this point was the same as that of TEB cell

cultures, except the TEF cells were resuspended in BME (5%) to a final concentration of 1 x 10^6 cells/ml.

Three ml of the BME-TEF cell suspension was added to each sterile 35 mm^2 plastic culture plate.

Chicken Embryo Fibroblast (CEF) Cells

CEF cells were used to determine plaquing ability of various strains of Sendai virus. The cultivation procedure was the same as for TEF cells except 10 to 12 day-old fertile chicken eggs were used.

Plaque Assay

The Sendai virus strain to be assayed was filter sterilized by passing the allantoic fluid through a 0.45 μm filter (Gelman). The allantoic fluid was aseptically serially diluted in BSS and 0.2 ml of each dilution was added to a confluent monolayer of either TEF or CEF cells depending on the system being tested. The virus was allowed to attach at room temperature for 30 min and overlay medium (2 ml) was added to each plate. Plates were incubated at 31°C for 48 hr at which time neutral red overlay (2 ml) was added. Plaques were observed by 60 hr post-infection.

Hemagglutination Titration (HA)

Hemagglutination titers of Sendai virus were determined in plastic disposable trays. PBS (0.2 ml) was added to 12

wells of the tray. A virus sample (0.2 ml) was added to the first well and a serial two-fold dilution was made. An equal volume of washed chicken red blood cells (0.6%) suspended in PBS was then added to all wells as an indicator. The trays were incubated for 1 hr at room temperature. The viral HA titer was considered to be the inverse of the dilution of virus in the well showing 50% hemagglutination.

Hemagglutination Inhibition Assay (HAI)

Turkey serum samples were tested to determine if they contained antibodies against Sendai virus. The procedure followed is described in the Manual of Clinical Immunology (23).

Hemadsorption Assay (HAD)

Modification of plasma membranes of TEB cells by Sendai virus was detected by HAD assays. Growth media from monolayered TEB cells infected with Sendai virus was decanted and the cells washed with BSS. The cells were then detached from the culture flask by addition of trypsin-EDTA. Upon completion of detachment, the cells were pelleted at 1000 x g for 5 min. The cells were resuspended in a small volume of PBS by vortexing. One drop of a cell suspension was placed on a clean microscope slide and allowed to air dry. The slide was fixed in 95% ethanol for 30 sec and allowed to

air dry. The slide was then saturated with a chicken red blood cell (30%) solution and incubated in a moist chamber for 30 min at room temperature. The slide was washed in three changes of PBS and observed under the microscope.

Plasma Membrane Isolation

The plasma membranes, both viral modified and unmodified, were isolated as follows: growth medium was decanted from culture vessels containing monolayered TEB cells. The vessels were rinsed gently with Tris swelling buffer (20 ml). The rinse was removed and replaced by fresh Tris swelling buffer. The monolayers were bathed in this solution for 30 min at room temperature. The swollen cells were then transferred to 4°C for 30 min to rupture the cells. The sheets of membranes and cells still attached to the culture vessel were scraped off with a rubber policeman and collected in large Nalagene centrifuge bottles. The bottles were centrifuged at 10,000 x g for 45 min at 4°C to pellet all large particulate material. The pellet was resuspended in a minimal amount of cold Tris swelling buffer and homogenized by aspirating the solution vigorously through a 21-gauge needle until microscopic examination indicated that all the cells had been disrupted. The membrane suspension was then layered onto discontinuous sucrose gradients which were composed of 60% (5 ml), 45% (10 ml), 30% (10 ml), and 15% (5 ml) sucrose carefully layered on top of each other in a cellulose nitrate ultracentrifuge tube. The gradients

were centrifuged for 90 min at 72,000 x g at 4°C. Upon completion of centrifugation the gradients were observed to contain an opaque fluffy band at the interface between the 30% and 40% bands. This material was harvested and then diluted in 3 to 4 times its volume of PBS. The membranes were then pelleted by centrifugation at 80,000 x g for 1 hr at 4°C. The pellet was resuspended in a minimal amount of PBS, aspirated through a 21 gauge needle, and layered onto a second discontinuous sucrose gradient. The gradients were centrifuged as above, and the same opaque zone was harvested. The membranes were diluted in 3 to 4 volumes of PBS and pelleted. The pellet was resuspended in a minimal amount of PBS, aspirated through a 21 gauge needle and a protein determination done by the method of Yonetani (35). The membranes were then diluted to contain 1 mg of protein/ml of solution and frozen at -35°C.

Procedure for Preparation of Virus Modified TEB Cells and Modified Membranes

Virus modified TEB cells used in lymphocyte adherence assays, fluorescent antibody studies and for isolation of membranes for injection were prepared by the following procedure. The MEM (10%) growth medium was carefully decanted from the culture vessel, and a suspension of virus diluted in MEM (10%) to 300 HAUs per ml was added in the following volumes: roller bottles (8 ml), 75 cm^2 flasks (3 ml).

The monolayers were then bathed in this limited volume for 30 min at room temperature. Growth medium was then added to the pre-infection volume and the monolayers were incubated at 31°C for 12 hr. Modified membranes were isolated by the method described for plasma membranes. Membranes to be injected into turkeys were exposed to UV for 20 min to inactivate any infectious Sendai virus. Infectivity was checked by inoculating 10 day old embryonated chicken eggs with such membranes, incubated for 48 hr and allantoic fluid was tested for ability to hemagglutinate chicken RBCS.

Polyacrylamide Gel Electrophoresis (PAGE)

Slab PAGE was used to observe differences in the protein composition of OSU-T Sendai and VR-105 Sendai obtained from the American Type Culture Collection. Slab PAGE was also used to monitor the viral alteration of TEB cells infected by Sendai virus and control TEB cells. The gels were constructed to give a running gel length of 20 cm, a stacking gel of at least 1 cm and a sample well-forming gel which contained 10 wells of 2 cm length by 1 cm width. The running gel was composed of 7.5% acrylamide as follows: stock solution acrylamide (6.25 ml), water (12.44 ml), lower Tris buffer (6.25 ml), 10% ammonium persulfate (62.5 µl), and TMED (5 µl). The solution was thoroughly mixed and pipeted into the gel from the top. n-Butanol was layered on top of the gel before polymerization to act as a leveler. After

polymerization the butanol was removed by successive washing with isopropanol and water and was then dried. The stacking gel was composed of 3.5% acrylamide as follows: stock solution of acrylamide (1.1 ml), upper Tris buffer (2.0 ml), water (6.76 ml), 10% ammonium persulfate (30 µ), and TEMED (10 µl). This solution was mixed and layered onto the stacking gel. The entire gel was allowed to age at room temperature for 18 to 30 hours before use.

Virus samples were treated with SDS (1%), beta-mercaptoethanol (1%) and heat (boiling water bath for 2 min) before addition to the wells. Crystalline sucrose was used to give the samples density, and bromthymol blue was used as a tracking dye.

The gels were run at 12 to 15 mA using the reservoir buffer previously given. The gel was run in a 4°C chamber to prevent overheating. Electrophoresis was considered complete when the tracking dye had migrated 10-12 cm.

The gel was fixed in fixing solution at 54°C for 10 to 15 min. Gels were stained in Coomassie Brilliant Blue solution for 10-15 min at 54°C. The gel was destained for 1 hr in destaining solution. The gel was washed twice with water, and dried under vacuum on white poster board for observation of bands.

Indirect Fluorescent Antibody Assay

Detection of anti-normal TEB cell and anti-Sendai virus modified TEB cell immunoglobulin G (IgG) in turkey serum was determined by indirect fluorescent antibody assays.

Preparation of Antigen

Pure turkey IgG was obtained by non-specifically precipitating with 5 volumes of saturated ammonium sulfate slightly alkaline conditions (pH 8.0). The precipitate was collected by centrifugation at 1000 x g for 15 min and resuspended to half its original volume in PBS. This procedure was repeated twice with the final precipitate resuspended in half the original volume of the serum. The precipitate solution was dialyzed for 72 hr at 4^{o}C against PBS. The protein concentration was determined and the IgG solution diluted to 1 mg/ml and frozen in 1 ml aliquots.

Anti-Turkey Antibody Preparation and Fluorescent Labeling

Antibodies to turkey IgG were obtained by intravenously injecting rabbits with 1 mg of turkey IgG three times a week for 6 weeks. The hyper-immune rabbit serum was collected via cardiac puncture. The IgG fraction of the rabbit serum was isolated and purified as described above. The presence of anti-turkey IgG activity was shown by Ouchterlony double

diffusion testing. The rabbit source anti-turkey IgG was then labeled with FITC (24).

Cell Antigen Preparation

Normal and Sendai virus modified TEB cells were cultured as mentioned previously. Monolayered TEB cells were detached from the tissue culture flasks with BSS-EDTA (0.02%) solution and collected in a screw-cap tube. The cells were washed three times in PBS and suspended the final time to a concentration of 1.5×10^5 cells/ml in PBS. A clean microscope slide was then divided into two fields with one half labeled as normal cells and the other labeled as modified cells. To the corresponding side, one drop of either normal TEB cells or Sendai modified TEB cells was added. The slides were allowed to air dry before fixation in acetone for 5 min at $4^{\circ}C$. The acetone was allowed to evaporate and the slides stored at $-30^{\circ}C$.

Fluorescent Antibody Staining Procedure

The rabbit source anti-turkey IgG was diluted 1:10 in a solution of naphthalene black (0.01%) in PBS to reduce autofluorescence. The turkey serum to be tested was diluted 1:4 in PBS to reduce autofluorescence. A slide was removed from $-30^{\circ}C$ storage and placed in a moist chamber. The diluted serum to be tested was added to both normal and modified cells and the slide was incubated at $37^{\circ}C$ for 30

min. The slide was rinsed with three 10 min washings of PBS, air-dried, and returned to the moist chamber. The slide was saturated with fluorescently labeled rabbit anti-turkey IgG IgG and incubated for 30 min at 37°C. The slide was again rinsed with three 10 min washes of PBS, plus a 1 min rinse in water to remove phosphate crystals. The slide was air-dried and observed. A positive fluorescent control was obtained by direct fluorescent labeling of modified TEB cells with FITC-labeled anti-Sendai virus rabbit IgG. The rabbit anti-turkey IgG added directly to non-infected TEB cells was used as a negative control. Serum samples were judged as positive for fluorescence or ngegative for fluorescence for both cell types.

Lymphocyte Adherence Test (LAD)

LAD was performed on all turkeys during the 20th week to detect the presence of lymphocytes sensitized to either normal TEB cells or Sendai-modified TEB cells. Turkey blood (4 ml) was drawn by inserting an eighteen gauge needle into the major wing vein and collecting the blood in a screw-capped test tube containing Alsever's solution (4 ml). The tubes were inverted to mix and placed on ice for transport. Lymphocytes were isolated by layering citrated turkey blood (8 ml) gently on top of Histoplaque 1077 (3 ml, Sigma) and centrifuging at 400 x g for 30 min in a swinging bucket clinical centrifuge. The buffy coat at the

histoplaque-serum interface which contained the lymphocytes was carefully collected with a pasteur pipet and added to a screw-cap tube containing ammonium chloride (0.87%, 10 ml). The lymphocytes were incubated in the ammonium chloride solution for 5 min at room temperature to lyse any residual red blood cells present. The lymphocytes were pelleted by centrifugation at 250 x g for 10 min. The supernatant was poured off and the lymphocytes resuspended in BSS (10 ml). The lymphocytes were washed three more times before being suspended to a concentration of 1.5×10^6 cells/0.5 ml. The lymphocyte suspension (0.5 ml) was then incubated with either normal TEB cell suspension (0.5 ml) or with modified TEB cell suspension (0.5 ml) for 30 min at 37°C. The mixture was then concentrated by centrifugation at 200 x g for 3 min. The supernatant was removed and the cells gently resuspended in BSS (0.1 ml). A wet mount was prepared and examined under phase contrast microscopy. Three hundred cells were then observed and the slide was determined to be positive if 3 or more lymphocytes were adhered to a cell.

Complement Fixation Test (CF)

Complement fixation testing was used to detect the presence of anti-normal TEB cell and anti-Sendai virus modified TEB cell antibodies in turkey serum samples. Fresh guinea pig complement was obtained just prior to use and was stored on ice or frozen in 0.2 ml aliquots. Anti-sheep red

blood cell antibody hemolysin was obtained by injecting a suspension of sheep red blood cells (1%) into the marginal ear vein of rabbits every other day for three weeks. The rabbits were rested one to two weeks before being bled by cardiac puncture. The hemolysin was heat-inactivated at 56°C for 30 min to destroy naturally occurring complement. The proper concentrations of hemolysin and complement to be used in the assay were determined in the standard procedure of the Manual of Clinical Immunology (23).

Crude membrane suspensions of Sendai virus modified TEB cell membranes were used in conjunction with known positive anti-Sendai virus antibody to determine the optimal antigen concentration. The assay was then run as given in the Manual of Clinical Immunology (23) using heat-inactivated turkey serum samples as the test serum.

CHAPTER III

RESULTS

Comparision of OSU-T Sendai to ATCC VR-105 Sendai

The strain of Sendai virus referred to as OSU-T Sendai was isolated by Dr. Mark Sanborn at Oklahoma State University in 1975. Sanborn began passaging Sendai virus in turkey eggs. After several passages it was observed that the turkey source Sendai virus would form plaques on a monolayer of chicken embryo fibroblasts, whereas Sendai virus passaged in chicken eggs did not plaque monolayered chicken embryo fibroblasts. OSU-T Sendai was isolated by removing a single plaque from the monolayer of turkey embryo fibroblasts and using the virus present to infect fertile turkey eggs. The virus was plaque purified three times. The virus has been passaged in turkey eggs since that time.

The question raised by the isolation of a strain of Sendai virus which would plaque chicken embryo fibroblasts was two-fold. First what other physical and morphological differences did OSU-T Sendai exhibit in comparision to the standard strain of Sendai virus? Secondly, and most importantly, was this modification of the virus particle a host-induced change which will revert back to the standard

characteristics upon back passage of the virus in chicken eggs, or has there been a genetic mutation or change in the virus which will not change upon such back passage of the virus in chicken eggs?

The morphology of OSU-T Sendai virus and the standard strain of Sendai virus, VR-105, obtained from the American Type Culture Collection, is similar in size and shape as observed in Figure 2. OSU-T and VR-105 Sendai demonstrate the ability to agglutinate chicken red blood cells when incubated at room temperature. Both virus strains were observed to cause hemolysis of chicken red blood cells when the incubated temperature was raised to 37°C for 30 min. Hemagglutination was inhibited in both strains of virus when incubated in the presence of antiserum obtained by the immunization of rabbits with OSU-T Sendai. Both virus strains were found to replicate well at 31°C.

To determine if the ability of OSU-T Sendai virus to form plaques on a monolayer of chicken fibroblasts is host-dependent or host-independent, OSU-T Sendai virus was back-passaged in chicken eggs four times. The results of this plaquing experiment are shown in Table II which demonstrates that the plaque forming ability is not lost as OSU-T Sendai is back-passaged in chicken eggs. The plaque forming units (PFUs) per Hemagglutination unit (HAU) for OSU-T Sendai was 1.40×10^6. The PFU/HAU ratio did seem to decrease during the back passage to 8.51×10^4 for the fourth passage of OSU-T in chicken eggs. The plaques were

Figure 2. Electron micrographs of VR-105 Sendai virus, OSU-T Sendai virus, and OSU-T Sendai after passaging 3X in chicken eggs.
A-VR-105 Sendai virus
B-OSU-T Sendai virus
C-OSU-T Sendai virus
D-OSU-T Sendai virus passaged 3X in chicken eggs
E-OSU-T Sendai virus passaged 3X in chicken eggs

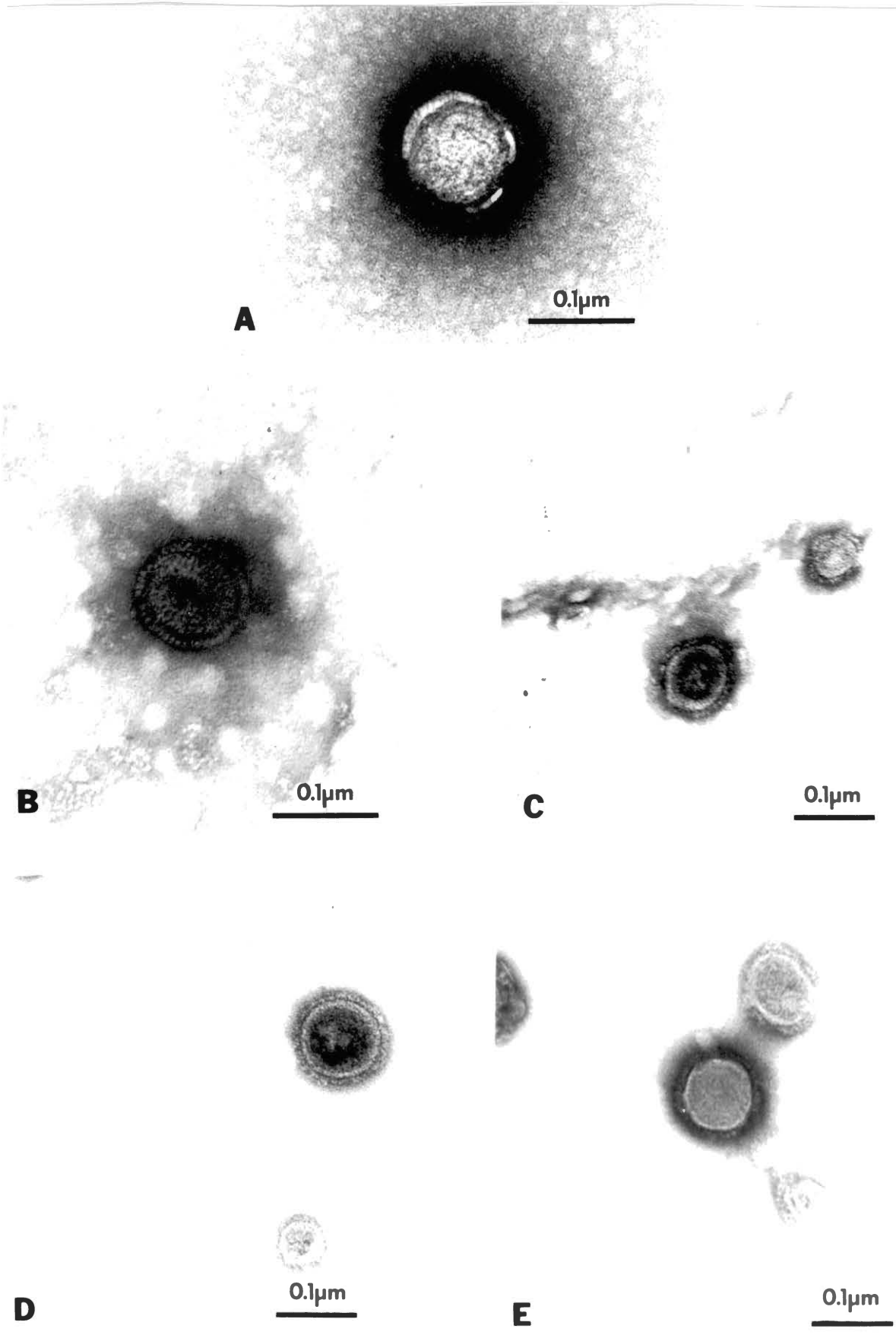
A
0.1µm
B
0.1µm
C
0.1µm
D
0.1µm
E
0.1µm

TABLE II

PLAQUING EFFICIENCY ON CHICKEN EMBRYO FIBROBLASTS OF TURKEY SOURCE SENDAI AFTER PASSAGE IN CHICKEN EGGS

Passage Number	PFU'S/ML	HAU'S/ML	PFU'S/HAU
Turkey Source	4.52×10^{8}	320	1.40×10^{6}
1	3.82×10^{10}	1280	2.98×10^{7}
2	6.30×10^{5}	1280	4.92×10^{2}
3	4.90×10^{7}	4690	1.19×10^{4}
4	5.45×10^{7}	640	8.51×10^{4}

yell formed at 48 hr post-infection and were 1 to 2 mm in diameter (Figure 3).

VR-105 Sendai virus, which had been passaged 32 times in chicken eggs at the American Type Culture Collection, was passaged 3 times in turkey eggs to determine if plaque formation could be observed on chicken embryo fibroblasts. The VR-105 Sendai virus was found, as expected, to be unable to form plaques on chicken embryo fibroblasts. After each passage in turkey eggs, the PFU/HAU ratio increased slightly (Table III). These plaques, however, were much lighter than the plaques formed by OSU-T Sendai and were not observable after 72-96 hr post-infection.

The ability of OSU-T Sendai, back-passaged in chicken eggs, to plaque turkey embryo fibroblasts was also tested (Table IV). The results were similar to those found when OSU-T Sendai was back-passaged in chicken eggs and plaqued on chicken embryo fibroblasts. The OSU-T Sendai had a PFU/HAU ratio of 8.00×10^6. This value decreased to 1.32×10^3 PFU/HAU after passage of the virus four times in chicken eggs. This reduction may also be due to defective interfering particles (DI). The plaques again were observed clearly in 48 hr post-infection (Figure 3).

As indicated in Table V, the VR-105 endai did not plaque turkey embryo fibroblasts (Figure 3). The VR-105 Sendai virus that was passaged in turkey eggs three times did not plaque turkey embryo fibroblasts, even after 96 hr incubation.

Figure 3. Demonstration of plaque forming ability of different types of Sendai virus.
A. Turkey source Sendai virus on turkey embryo brain cells
B. Turkey source Sendai virus on turkey embryo fibroblasts
C. OSU-T Sendai passaged 3 times in chicken eggs on turkey embryo fibroblasts
D. OSU-T Sendai on chicken embryo fibroblasts

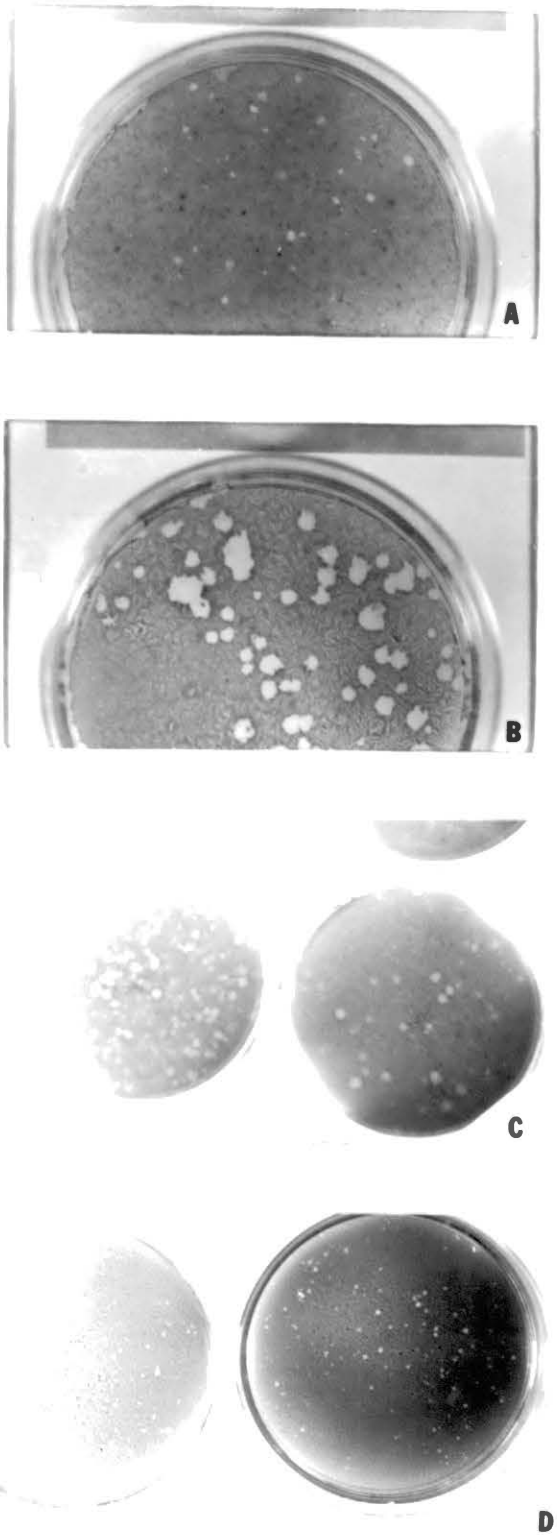
A
B
C
D

TABLE III

PLAQUING EFFICIENCY ON CHICKEN EMBRYO FIBROBLASTS OF CHICKEN SOURCE SENDAI (ATCC VR-105) AFTER PASSAGE IN TURKEY EGGS

Passage Number	PFU'S/ML	HAU'S/ML	PFU'S/HAU
ATCC VR-105	0	640	0
1	1.8×10^2	80	2.25
2	2.2×10^5	2560	8.59×10^1
3	3.1×10^6	5120	6.05×10^2

TABLE IV

PLAQUING EFFICIENCY ON TURKEY EMBRYO FIBROBLASTS OF TURKEY SOURCE SENDAI AFTER PASSAGE IN CHICKEN EGGS

Passage Number	PFU'S/ML	HAU'S/ML	PFU'S/HAU
Turkey Source	5.12×10^9	640	8.00×10^6
1	3.02×10^8	640	4.72×10^5
2	2.50×10^8	1280	1.95×10^5
3	2.45×10^6	1280	1.91×10^3
4	1.69×10^6	1280	1.32×10^3

TABLE V

PLAQUING EFFICIENCY ON TURKEY EMBRYO FIBROBLASTS OF CHICKEN SOURCE SENDAI (ATCC VR-105) AFTER PASSAGE IN TURKEY EGGS

Passage Number	PSU'S/ML	HAU'S/ML	PFU'S/HAU
ATCC VR-105	0	320	0
1	0	80	0
2	0	1280	0
3	0	640	0

The effect of OSU-T Sendai and VR-105 Sendai on primary, secondary and tertiary passaged chicken embryo fibroblast cells was studied to determine if plaquing ability is lost as cells are passaged. OSU-T Sendai did not lose its ability to form plaques on passaged chicken embryo fibroblasts (Table VI). The VR-105 Sendai did not form plaques on passaged chicken embryo fibroblasts (Figure 3).

Polyacrylamide gel electrophoresis (PAGE) was used to determine any differences in protein composition of OSU-T Sendai, VR-105 Sendai, and OSU-T Sendai passaged three times in chicken eggs. Approximate molecular weights of individual proteins were determined by running protein standards (Sigma). Figure 4 illustrates the banding patterns of each of the virus samples and a protein standard (Sigma Chemical Co.) after they were treated with 1% SDS, 1% beta-mercaptoethanol, and boiled for 3 min. The number of proteins and their molecular weights are given in Table VII. The protein composition of OSU-T Sendai is identical to that of OSU-T Sendai passaged three times in chicken eggs. The protein composition of these two samples, however, differed from the composition of VR-105 Sendai. VR-105 contains an 81,000 dalton protein which the other two samples do not appear to contain. On the other hand, VR-105 does not contain the 31,000 dalton protein that can be observed in the other two OSU-T samples. The 160,000 dalton protein band was not always observed in the VR-105 Sendai, but was always present in the OSU-T samples.

TABLE VI

PLAQUING EFFICIENCY OF TURKEY SOURCE SENDAI VIRUS ON PRIMARY, SECONDARY, AND TERTIARY PASSAGE CHICHEN EMBRYO FIBROBLASTS

Cell Passage	PFU'S/ML	HAU'S/ML	PFU'S/HAU
1	4.52×10^8	320	1.4×10^6
2	8.0×10^7	80	1.0×10^6
3	4.0×10^3	6409	6.25×10^{10}

PLAQUING EFFICIENCY OF CHICKEN SOURCE SENDAI VIRUS (ATCC VR-105) ON PRIMARY, SECONDARY, AND TERTIARY PASSAGE CHICKEN EMBRYO FIBROBLASTS

Cell Passage	PFU'S/ML	HAU'S/ML	PFU'S/HAU
1	0	320	0
2	0	80	0
3	0	5120	0

Figure 4. Polyacrylamide gel electrophoresis of viral proteins of OSU-T and VR-105 Sendai virus.
C - VR-105 Sendai
T - OSU-T Sendai
HMWP = High molecular weight protein
P = P protein
HN = Hemagglutinating Neuraminidase Protein
NP = Nucleoprotein
F = F protein
M = Matrix protein

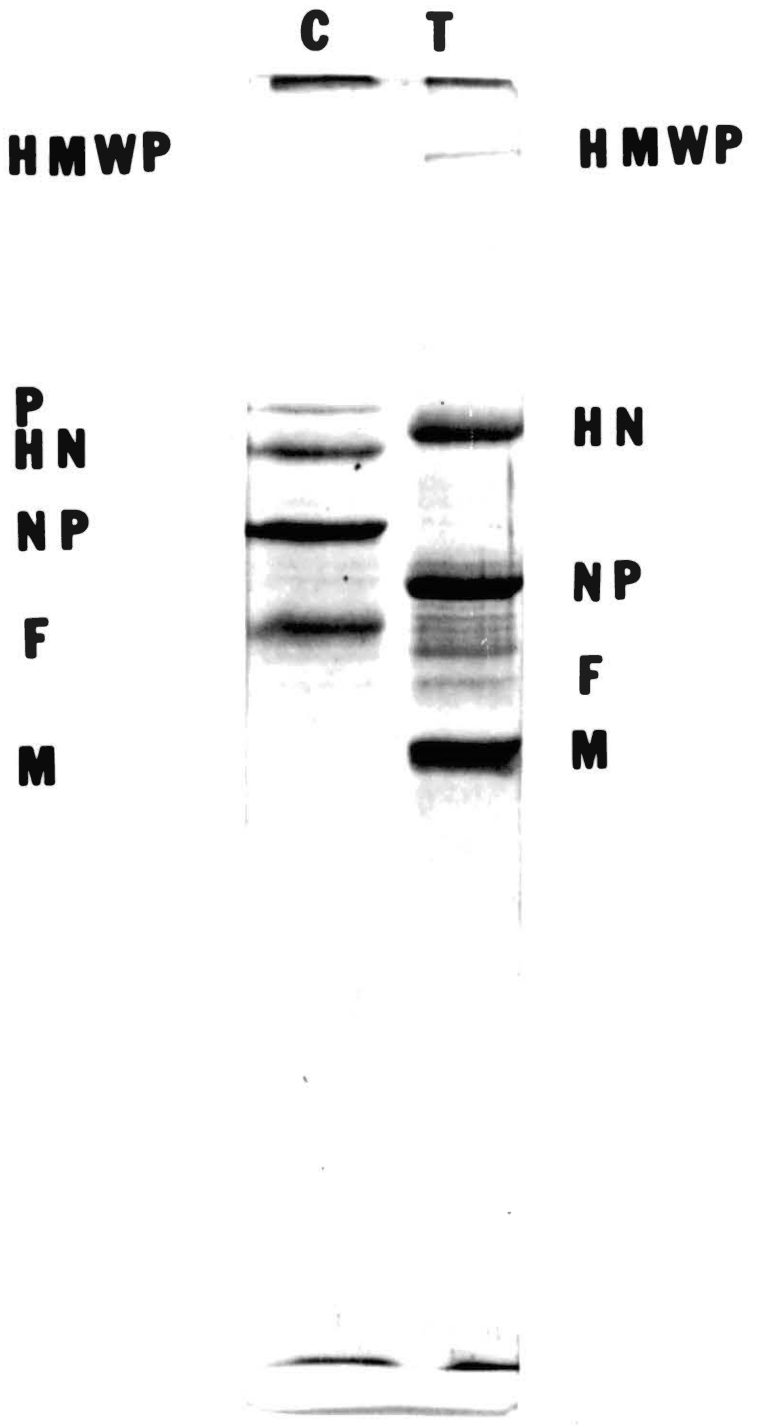
C
T
HMWP
HMWP
P
HN
NP
F
M
HN
NP
F
M

TABLE VII

COMPARISON OF MOLECULAR WEIGHTS OF PROTEINS OF OSU-T AND VR-105 SENDAI VIRUSES

Protein	Molecular Weight (daltons)		
	OSU-T	OSU-T[a]	VR-105
1	160,000	160,000	160,000
2	77,000	77,000	81,000
3	52.000	52,000	75,000
4	48,000	48,000	51,000
5	45,000	45,000	48,000
6	44,000	44,000	45,000
7	43,000	43,000	43,000
8	40,000	40,000	
9	31,000	31,000	

[a]OSU-T back passaged in chicken eggs three times

Experimentation with Turkeys

In this investigation the determination as to whether an autoimmune disease was induced into White Hollin Turkeys was based on results from three areas. The first was direct weekly observation of the turkeys. This included testing the ability of the birds to "right" themselves when placed on their backs and recording the weekly weight gain of each bird. The second area was examination of the possible role of humoral substances in the blood of each turkey. The possible existence of anti-self antibodies in the serum of each turkey was tested by complement fixation and fluorescent antibody procedures, and the presence of antibodies directed against Sendai virus was tested by hemagglutination inhibition assays. The final area of interest was to determine whether the turkeys demonstrated a cellular response. The response was examined by lymphocyte adherence tests.

The results of the weekly test of "righting reflex" are listed in Tables VIII and IX. Table VIII contains the observations for turkeys which received normal TEB membranes. Except for birds 63, 64, 69, and 79, the group did not show any major defect in the "righting response". Bird 63 was essentially normal until a non-neurological leg injury developed. This leg injury was directly responsible for the bird's inability to get up. The bird's condition deteriorated rapidly due to her inability to protect herself from attacks from other birds. Birds 64 and 69 seemed to show

TABLE VIII

OBSERVATION OF RIGHTING REFLEXES FOR CONTROL GROUP OF TURKEYS

Bird	Sex	Week 2	4	6	8	10	12	14	16	18	20	FINAL
55	F	0	0	0	0	0	+	0	0	0	++	0
56	M	0	0	0	0	0	0	0	+	0		0
57	F	0	0	0	0	0	+	0	0	0	0	0
58	M	0	+	0	0	+	0	0	0	0	0	0
59	F	0	0	0	++++	0	0	0	0	0	0	0
60	M	0	0	0	0	0	0	0	0	(++)	0	0
61	M	0	0	0	0	0	0	0	+	0	0	0
62	F	0	0	0	++	0	0	+	0	0	0	0
63	F	0	0	0	0	++++	++++	++++	+++	++++	-	-
64	M	0	0	+	++	0	+++	0	0	0	0	++++
65	F	0	0	+	0	0	0	0	+	0	0	0
66	F	0	0	0	0	0	0	0	0	0	0	0
67	F	0	0	0	0	0	0	0	0	0	0	0
68	F	0	+	+	0	0	+	0	0	0	0	0
69	M	0	0	0	0	+	++	+++	0	0	0	0
71	F	0	0	0	0	0	0	0	0	0	0	0
72	F	0	0	0	0	0	0	0	0	0	0	0
73	M	0	0	0	0	+	0	0	+	0	0	0
79	M	0	+	0	++	++	++++	+++	+++	++++	++++	++++

0 = No problem
+ = Slight stumble upon turning
++ = Slight problem turning over
+++ = Definite problems turning over and stumbling upon turning
++++ = Unable to turn over
- = Bird died

TABLE IX

OBSERVATION OF RIGHTING REFLEXES FOR EXPERIMENTAL GROUP OF TURKEYS

Bird	Sex	Week 2	4	6	8	10	12	14	16	18	20	FINAL
80	F	0	++	++++	++++	++++	++++	++++	++++	++++	++++	++++
82	F	0	0	0	0	+	0	++	0	0	++	++
83	M	+	++	+	++	0	++	++	+++	0	+++	0
84	F	0	+++	0	+++	0	+	+++	0	+++	+++	+++
85	M	0	+	0	0	0	+	0	+	0	0	0
86	F	0	0	0	0	++	++	++	++	+++	0	0
87	F	0	0	0	++	++	+	++	0	0	0	++
88	M	0	0	+	0	+	0	+	+	++	0	+++
89	F	0	+++	0	0	0	+	+	+++	+++	0	0
90	F	0	0	0	+	+	0	++	0	+++	0	0
92	M	0	0	0	0	0	0	++	++	0	0	0
93	M	0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
94	M	0	0	0	0	0	++	0	0	0	0	0
96	F	0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
97	M	0	0	0	0	0	0	+	++++	0	++++	++++
98	F	0	0	0	0	+	0	++	++	+++	0	0
99	F	0	0	0	0	0	0	0	0	0	0	0

0 = No problem
+ = Slight stumble upon turning
++ = Slight problem turning over
+++ = Definite problems turning over and stumbling upon turning
++++ = Unable to turn over
- = Bird died

short periods of significant neurological disfunctions for no explainable reason; however, they seemed to return to normal functioning. Bird 79 gradually developed neurological disfunction for unknown reasons and never recovered. Most control birds had little or no observable neurological problems.

The experimental group of birds injected with TEB cell membranes modified with Sendai virus showed marked neurological disfunction. All birds except 99 showed at least some neurological disturbance. Birds 80 and 93 demonstrated severe impairment of neurological function. Bird 80 was unable to right herself after the sixth week. Bird 93 demonstrated uncontrollable head shaking, trouble with eye focusing, and had difficulty getting up when placed on his back. Most of the other birds in the experimental group showed less pronounced disfunction, but definitely had greater disfunction than the control birds.

The mean weight gain for control hens was found to be 5.2025 kilograms, whereas the mean weight gain was found to be 4.9365 kilograms for experimental hens. The mean weight gain for control toms was found to be 9.113 kilograms, whereas the weight gain for experimental toms was found to be 8.717 kilograms. In most cases the birds with the least weight gain demonstrated the most significant neurological disturbances (Tables X and XI).

The search for humoral agents in the serum containing anti-TEB cell and anti-TEB cell modified with Sendai virus

action used the complement fixation and fluorescent antibody tests. Complement fixation revealed no complement binding antibodies to either TEB cells or TEB cells modified with Sendai virus. The test was repeated three times with the proper controls reading as expected.

The results of the fluorescent antibody procedures also indicated the absence of circulating antibodies either to TEB cells or to TEB cells modified with Sendai virus. There were no observable differences between negative control, positive control, and the serum samples being tested during double blind examination. The positive control serum was suspected of being of low titer.

The serum samples of each turkey were tested to determine their hemagglutination inhibition titer. The results for each control bird are given in Table XII and the results for each experimental bird in Table XIII. The mean HAI titers for each week are listed in Table XIV for both control and experimental birds.

Lymphocytes isolated from each bird were used in a lymphocyte adherence (LAD) test to determine if there was a difference between the cellular immune response of experimental birds when compared to the control birds. The test was conducted as a blind experiment to avoid any prejudice in the determination. The results are given in Table XV. The mean percent LAD for control birds was 3.85, and 2.94 for the experimental birds.

TABLE X

COMPARISON OF WEIGHT GAIN AND DISEASE INDEXES OF HENS

Weight (Grams)	Bird	Disease Index[a]
3594	96	29
3968	80	38
4521	65	2
4619	89	11
4726	99	0
4821	90	7
4824	84	19
4840	68	3
5090	59	4
5096	72	0
5178	67	0
5235	55	3
5245	82	7
5303	72	0
5404	86	11
5478	57	1
5604	62	3
5680	66	0
5973	87	9
6182	98	8

[a]Disease index was determined by assigning 1 point values for each (+) given to the bird in Tables VIII-IX.

TABLE XI

COMPARISON OF WEIGHT GAIN AND DISEASE INDEXES OF TOMS

Weight (grams)	Bird	Disease Index[a]
5822	93	30
5962	64	10
7453	79	27
8397	61	1
8508	56	1
8549	83	16
8676	73	2
8709	88	9
9014	97	13
9114	94	3
9870	58	2
9880	85	3
9931	92	4
10611	60	2
13427	69	6

[a]Disease index was determined by assigning 1 point values for each (+) given to the bird in Tables VIII-IX.

TABLE XII

HAI TITER OF TURKEYS INJECTED WITHOUT VIRUS INFECTED BRAIN CELLS

Turkey	Week									
	3	4	6	8	10	12	14	16	18	20
55	80	640	160	20	40	20	10	10	0	-
56	160	160	ns	ns	160	40	20	20	0	20
57	80	80	ns	ns	160	1280	160	160	80	640
58	ns	40	ns	80	640	40	40	20	40	160
59	640	80	ns	ns	640	20	20	10	10	20
60	320	160	ns	40	80	10	10	10	0	0
61	40	640	80	ns	80	20	20	20	10	20
62	40	80	160	ns	320	160	80	20	20	80
63	160	320	ns	5120	320	640	160	80	-	-
64	80	320	160	640	640	160	1280	0	20	0
65	80	160	160	160	160	5120	20	20	20	40
66	320	160	5120	5120	5120	80	40	30	20	40
67	40	160	320	160	320	80	40	160	20	40
68	80	ns	80	ns	640	160	40	80	20	20
69	80	80	80	ns	80	10	10	0	0	0
71	640	80	80	2560	5120	1280	320	320	160	40
72	40	80	40	20	20	80	40	40	10	10
73	640	20	40	80	80	160	40	40	160	40
79	320	160	160	160	160	ns	0	0	0	0

ns = No serum available; - = Turkey died

TABLE XIII

HAI TITER OF TURKEYS INJECTED WITHOUT VIRUS INFECTED BRAIN CELLS

Turkey	Week									
	3	4	6	8	10	12	14	16	18	20
80	640	-	-	-	0	20	10	10	10	0
82	80	80	-	-	160	20	10	10	0	40
83	80	160	80	-	80	20	20	20	10	10
84	80	80	160	160	-	80	40	40	640	80
85	5120	80	1280	40	0	40	40	20	0	20
86	320	5120	640	-	40	40	20	20	10	10
87	40	640	40	-	20	40	20	20	10	20
88	80	160	40	40	40	20	20	320	10	0
89	-	320	40	40	40	80	20	80	10	20
90	80	80	80	80	320	80	20	640	10	20
92	320	640	320	160	160	80	40	640	40	20
93	80	640	640	320	-	320	40	80	10	20
94	80	80	-	320	160	160	20	80	10	10
96	80	320	-	2560	640	80	80	80	10	0
97	40	80	-	80	40	20	0	10	0	0
98	640	640	-	80	1280	40	20	40	20	20
99	-	80	1280	40	40	40	20	40	10	10

TABLE XIV

AVERAGE TITER (HAI) FOR TURKEYS INJECTED WITHOUT VIRUS INFECTED BRAIN CELLS

	Week									
	3	4	6	8	10	12	14	16	18	20
Average Titer	4.72	4.72	4.85	5.33	5.33	3.94	3.00	2.44	1.77	2.30
Average Deviation	1.41	1.27	1.77	2.84	2.17	2.18	1.94	1.85	1.63	1.86

AVERAGE TITER (HAI) FOR TURKEYS INJECTED WITH VIRUS INFECTED BRAIN CELLS

	3	4	6	8	10	12	14	16	18	20
Average Titer	4.93	5.50	5.09	4.58	3.80	3.29	2.05	3.29	1.35	1.47
Average Deviation	1.90	1.75	2.30	1.78	2.77	1.16	0.96	1.93	1.62	1.12

TABLE XV

COMPARISON OF PERCENT LAD OF EXPERIMENTALS AND CONTROLS[a]

Controls		Experimentals	
Bird	Percent LAD	Bird	Percent LAD
55	13.5	80	3.5
56	0	82	9
57	2	83	5
58	1	84	0
59	8.5	85	0.5
60	1	86	0.5
61	0.5	87	0
62	7.5	88	4.5
65	7	89	0
66	0	90	2
67	2.5	92	5.5
68	5.5	93	7
69	4.5	94	1
71	5.5	96	4
72	0.5	97	3
73	3.5	98	4.5
79	3	99	0

[a]LAD = Lymphocyte Adherence

CHAPTER IV

DISCUSSION

Parameters of Biological Activity Affected by F_o Cleavage

The ability of Sendai virus to infect cells and form plaques has been related to the modification of virus proteins by proteolytic enzymes of the host (25, 18). This modification, according to Homma (19), is not heritable and the modified virus can be converted to the original form after a single passage in the original host. Chicken egg Sendai supports this point because chicken egg Sendai is infective for primary chicken and turkey embryo fibroblasts. However, the progeny virus produced by the embryonic fibroblasts are not infective because the embryonic fibroblasts lack the proteolytic enzyme found in the allantoic fluid which modifies the virus. The virus thus cannot infect neighboring cells in the monolayer, and therefore no plaques appear. If the culture medium is taken from infected fibroblasts and inoculated into chicken eggs, the virus will be modified by the allantoic fluid and infective particles will be produced. Thus a cycle of host-dependent modification is observed.

This host-dependent modification is, however, not

observed in OSU-T Sendai virus. OSU-T Sendai was found to plaque both chicken and turkey embryo fibroblasts with approximately equal efficiency (Tables II and IV). VR-105 Sendai virus obtained from the American Type Culture Collection and used as a control did not plaque either cell type. OSU-T Sendai was then passaged back through chicken eggs to determine if the ability to plaque would be lost without a change of host. OSU-T did not lose its ability to plaque chicken and turkey fibroblasts with successive passages in chicken eggs (Tables II and IV). The plaquing efficiency of OSU-T Sendai seemed to decrease upon back passage in chicken eggs; however, this reduction may be the result of a higher concentration of defective interfering particles (DI), which raise the HAU/ml concentration, but are not infective (17).

In order to determine whether the ability of OSU-T Sendai virus to plaque chicken and turkey embryo fibroblasts was the result of a single mutation, or the result of a permanent modification of the virus by the host cells, VR-105 Sendai virus was passaged in turkey eggs. It was observed that the VR-105 virus would begin to plaque chicken embryo fibroblasts to a limited extent after one passage in turkey eggs, and the efficiency seemed to increase with each successive passage (Table III). These plaques required two days longer to be observable, and even then were extremely light. The plaquing efficiency of the VR-105 Sendai back passaged in turkey eggs was very low when compared to the plaquing efficiency of OSU-T Sendai. The VR-105 Sendai

virus was unable to plaque turkey embryo fibroblasts after being passaged three times in turkey eggs.

It is evident that OSU-T Sendai has undergone a phenotypic change which enables the virus to plaque both chicken and turkey embryo fibroblasts. Whether this phenotypic change is the result of a single one-time mutation is as yet unclear. If the phenotypic change is a single one-time mutation, passaging VR-105 Sendai virus in turkey eggs would not produce virus that plaqued turkey or chicken embryo fibroblasts. On the other hand, if the phenotypic change is induced by successive passage in turkey eggs, the phenotypic change should be inducible in VR-105 Sendai after several passages in turkey eggs. Thus the results obtained from plaquing OSU-T versus VR-105 passaged in turkey eggs are inconclusive.

As mentioned in the introduction, several sources have reported that the ability of Sendai virus to infect cells depends upon the cleavage of the inactive F_o glycoprotein into the active F_1 and F_2 glycoproteins. If this is correct, it would be expected that OSU-T Sendai virus and VR-105 Sendai virus from the allantoic fluid of chicken eggs would have the same protein composition. Polyacrylamide gel electrophoresis of OSU-T Sendai and chicken allantoic VR-105 Sendai virus, however, reveals significant differences between the two (Fig. 4). Table XVI illustrates these differences and lists the designations and molecular weights which have been assigned to the major proteins in the liter denature. OSU-T Sendai virus has been

TABLE XVI

COMPARISION OF MOLECULAR WEIGHTS OF PROTEINS OF OSU-T AND VR-105 SENDAI VIRUSES

Protein	OSU-T (dal)	Designation	VR-105 (dal)	Designation	Literature (dal)	Designation
1	160,000	HMWP	160,000	HMWP	145,000	HMWP
2	77,000	HN	81,000	P	81,000	P
3	52,000	NP	75,000	HN	75,000	HN
4	48,000		51,000	NP	62,000	NP
5	45,000		48,000		57,000	F
6	44,000		45,000	F	50,000	
7	43,000		43,000		46,000	
8	40,000	F			43,000	
9	31,000	M			34,000	

HMWP = High Moleuclar Weight Protein
P = Protein
NP = Nucleoprotein
F = F Protein
M = Matrix Protein
HN = Hemagglutinating Neuraminidase Protein

shown to be completely lacking in the 81,000 dalton molecular weight protein, designated by the letter P. This protein is said to be involved in the RNA polymerase complex (30). VR-105 Sendai virus contains the P protein; but seems to possess below normal amounts of M protein, which is a major structural protein characteristically found in large amounts in Sendai viruses. OSU-T Sendai virus and VR-105 Sendai virus are also different in their composition of F protein and HN protein, which reportedly are required for adsorption and penetration (30). OSU-T Sendai's HN protein was consistently observed to be about 2,000 daltons heavier than the HN protein of VR-105 Sendai. The F protein of VR-105 Sendai, on the other hand, was observed to be 5,000 daltons larger than the F protein of OSU-T Sendai. This was not what one would expect of proteins which are supposed to be only modified by the host system proteolytic enzymes. If the only factor attributed to the modification and thus the activation of the F protein was the action of proteolytic enzymes in the allantoic fluid of eggs, then one would expect OSU-T Sendai and VR-105 Sendai to be identical. Thus the ability of OSU-T Sendai virus to plaque chicken and turkey fibroblasts is a host-independent event.

PAGE was also run on OSU-T Sendai virus which had been passaged three times in chicken eggs to determine how this back passage would affect the protein composition of the virus. The back passaged OSU-T was identical to OSU-T in

protein composition; therefore, no effects from such back passaging appear in PAGE for OSU-T Sendai virus.

The way to determine if OSU-T Sendai virus has been phenotypically changed to produce only the biologically active F_1 protein instead of the biologically inactive F protein is to observe the protein composition of OSU-T Sendai virus grown in cultured embryonic fibroblasts. Use of cultured cell-grown Sendai virus would eliminate the modification of viral proteins by proteolytic enzymes in the allantoic fluid of both chicken and turkey eggs.

In summary, it can be stated that OSU-T Sendai virus has undergone a genotypic change when compared to VR-105 Sendai virus. This change is expressed in the ability of OSU-T Sendai virus to plaque both chicken and turkey embryo fibroblasts, something which the VR-105 Sendai virus is unable to do. It is, however, able to infect them. This change in the OSU-T Sendai virus is heritable and host-independent. It is unclear whether this change is the result of a single mutation or the result of the host modification of the virus genome. OSU-T Sendai virus and VR-105 Sendai virus are substantially different in protein composition of those proteins reported to be important for infection and in several other proteins.

Induction of an Autoimmune Disease in Turkeys by Injection of Turkey Embryo Brain Cell Membranes Modified by Sendai Virus

The determination of whether an autoimmune disease was induced in the experimental group of turkeys was based on three criteria. First, did the experimental group of turkeys display clinically observable signs of neurological disturbance when compared to the control group of birds? Secondly, did birds in the experimental group contain antibodies in their serum which are not only active against brain cells altered by Sendai but against normal brain cells? Finally, did the experimental group exhibit an elevated cellular response directed against normal brain cells as well as brain cells modified by Sendai virus?

The observation of clinical signs indicating neurological disorder were primarily based on testing the "righting reflex" of each injected bird. This observation was totally subjective. The observations were not made in a blind manner, because of the location and housing of the birds. However, these experiments were done as objectively as possible, and in those cases where the righting reflex was missing entirely, there was little subjectiveness involved. Tables VIII and IX illustrated the righting reflex results for control and experimental birds. It is evident that the experimental group contained more birds with disturbances and, in most cases, the disturbance was more

severe than disturbances observed in the control birds. Forty-seven percent of the experimental birds were observed at the final examination to exhibit some neurological disorder, and seventy-five percent of this group were observed to have major neurological loss (+++ or ++++). Every experimental bird was observed at one time or another during the investigation to have some neurological disturbance, except bird number 99. Birds 80, 84, 93, 96 and 97 were all observed to have major neurological problems and, except for 97, accompanying physical problems. Those four birds were generally smaller than the rest of the birds of their sex and were being regularly pecked upon by the other birds in the group.

Starting early in the experiment, it was obvious that birds 80 and 93 were the two birds demonstrating the greatest degree of neurological disorder. Bird 80 was a small hen which regressed in the span of six weeks from normal responsiveness, to being unable to turn over after being placed on her back. The bird's condition never imporved from that point. Bird 93 never completely lost its righting reflex, but quickly developed difficulty in turning over. The bird could easily be picked out of the flock because of continuous tremors in the head and neck region. This bird was also unable to focus its eyes to peck at an object.

The control birds, on the other hand, were relatively free of major neurological disturbances. Only birds 79 and 64 were observed to have neurological oddities that could

not be explained. Occasionally, a control bird would demonstrate what appeared to be slight neurological disturbances; however, these incidents were never as numerous or as severe as those noted for the experimental group.

As mentioned, the average net weight gain for the control birds over the experimental birds was approximately 0.266 kilograms for hens and 0.395 kilograms for toms. This is an important statistic, possibly indicating the wasting symptom which has been associated with many autoimmune diseases (5). The birds in this experiment which exhibited the most chronic and pronounced neurological disorders were the birds which had the least weight gain (Tables X and XI). These differences in weight gain cannot be attributed to the difference in weight of the individual birds at the beginning of the investigation. Bird 80 was one of the heaviest birds at the beginning of the experiment, but finished as the second lightest.

The complement fixation test and fluorescent antibody staining procedure were used to determine if the serum of each bird contained antibodies against normal TEB cells as well as TEB cells modified by Sendai virus. No anti-TEB cell or anti-TEB cell modified with Sendai virus antibodies were detectable by the complement fixation test. The proper controls were always run to check the system. It is possible, of course, that avian antibodies will not bind guinea pig complement, causing the test to give erroneous negative results.

The fluorescent antibody procedure also did not indicate the presence of anti-TEB cell or anti-Sendai modified TEB cell antibodies. When this procedure was run as a blind experiment in which experimental birds sera (Birds 80, 93), control sera (Birds 56, 67), rabbit serum (negative control), and anti-Sendai virus antibody (positive control) were used to stain TEB cells modified with Sendai virus, no distinguishable differences could be observed. Unstained TEB cells were observed to have a high degree of autofluorescence.

The turkey serum samples were also tested to determine their hemagglutination inhibition titer. It would be expected that the experimental turkeys which received the Sendai virus modified virus membranes would have an elevated titer when compared to the control birds. This difference was not observed. There was no significant difference in the HAI titers between the experimental birds and the control birds (Tables XII, XIII, XIV, and XV). This unexpected result may be attributed to some unresolved problem with the assay system.

The experimental group also did not demonstrate an elevated cellular response. The results of the lymphocyte adherence tests revealed no significant differences between the experimental group of turkeys and the control group. In fact, the control group had a slightly higher LAD when the procedure was run in a blind assay.

It was observed that as a whole the experimental

group of birds demonstrated clinically significant neurological disorders as measured by the righting reflexes. The experimental birds were also shown to be lighter than their control counterparts. This is a characteristic of many autoimmune diseases. However, it was not possible to provide any immunological evidence to support the presumption that an autoimmune disease had been induced.

It is possible that with more sensitive techniques and less objective methods, such as ^{51}Cr release and possible a radioimmunoassay, evidence to support the clinical findings might be accumulated. Further, brains of the experimental group were collected at the termination of the experiment and are to be examined histologically for areas of demyelinization which would also be indicative of an autoimmune induced disease.

For further complement fixation studies, it would be advisable to obtain hemolysin by injecting chicken red blood cells in turkeys, and to use turkey complement instead of guinea pig complement; thus eliminating any problems of incompatibility between avian and mammalian complement binding systems. It has been postulated that birds are not immunocompetent until after 2-3 weeks of age, thus the entire experiment, if repeated, may be improved by beginning injections of turkeys at 1 month of age instead of two weeks, as was done here.

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