SUSCEPTIBILITY AND IMMUNOLOGIC RESPONSE OF ALLOXAN DIABETIC RATS TO VIRAL INFECTION

by

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ABSTRACT

In this study male albino rats of Sprague-Dawley strain were made diabetic and employed in a study to determine their susceptibility and immunologic response to the Lee strain of influenza B virus.

Diabetes was established by intravenous injections of alloxan. The animals were exposed to an aerosol containing the virus for specific time under controlled conditions of temperature and humidity. The lungs from the exposed animals were later removed and examined for gross pathology. A suspension of each lung was made and assayed for its viral content.

A group of animals simularly exposed were bled twenty-one days later and their serum antibody titer determined by the hemogglutination inhibition test.

The susceptibility and antibody production by the diabetic rats did not differ significantly from a simularly exposed non-diabetic group of control animals.

INTRODUCTION

The first recorded reference to diabetes was made in the Papyrus Ebers (Allen, Stillman and Fitz 1919; Striker 1961; Simowitz, 1962; Wrenshall, Hetenyi Jr., and Feasby 1963) a copy of Egyptian medical writing discovered by Ebers in 1862 in the tomb of Thebes in Egypt. However, convincing evidence indicates that the ancient Hindu medical men were familiar with the disease as described in the Work Chanaka Samhita which probably dates before the Papyrus. It is believed that diabetes as a clinical entity was known as early as 1550 B. C. The relatively late period of human history at which it was clearly recognized was probably due to the difficulty of distinguishing this disease from those of similar manifestations. It is generally accepted that Apollonius (230 B. C.) originated the word diabetes "going through" from the Greek words "dia" (through) and "betes" (flowing or going). The first accurate account of diabetes was given by Aretaeus of Cappadocia (A. D. 30-90). During the proceeding decades, various descriptions of and prescriptions for diabetes were given by Far Eastern and numerous European workers; Tehang Tchang-King (200 A. D.), Arabian: Rhazes (860-932 A. D.) Avicenna (980-1037 A. D.).

Paracelsus (1493-1541) noticed that the urine of a diabetic person on evaporation left a white powder. He thought this was salt and that it was responsible for the thirst of the kidneys and the cause of polyuria. A century later Thomas Willis Sidney (1621-1675), professor at Oxford University and physician to Charles II, made the important observation that the urine of diabetes was sweet "Wonderfully sweet as if imbued with honey or sugar" and later William Cullen (1709-1790) added the adjective "mellitus" from the Latin (honey sweet) to the name of the disease. It is of interest that the ancient Hindus had already noticed this fact much earlier, Charaka between 600 B. C. and 70 A. D. described "the urine in this variety looks like the expressed juice of the sugar cane . . . ants are attracted by the urine of persons afflicted."

The modern history of diabetes began in Liverpool, England in 1776. Mathew Dobson, a Yorkshireman, experimentally demonstrated the presence of sugar in diabetic urine. In the middle of the nineteenth century, a brilliant physiologist named Claude Bernard (1813-1878) discovered the mechanism of carbohydrate metabolism, glycogen, the glycogenic function of liver and an accurate method for quantitative determination of blood sugar. In his opinion, diabetes was due to overproduction of sugar by the liver. In 1809, the

Russian physiologist Oskar Minkowsky pancreatectomized a dog and to his surprise the animal became diabetic showing all the symptoms of a corresponding case in man. In 1901, Eugene Linsay Opie at John Hopkins University described destruction of the islets of Langerhans in a girl who died of diabetes. This prompted the British physiologist Sir Edward Sharpey Shafer, in 1916, to theorize that diabetes was due to a lack of an internal secretion by the islets which stimulated carbohydrate metabolism and he named this hypothetical secretion insulin. Previous workers, George Ludwig Zuelzer in 1908 and E. L. Scott in 1912, came very close to the successful demonstration of what Shafer later theorized. The dramatic discovery of insulin was made by Fredrick Grant Banting and Charles Herbert Best at the University of Toronto in 1921. These investigators isolated insulin, the blood glucose lowering component of the pancreatic islets. In January 1922, an eleven year old boy named Leonard Thompson was the first diabetic patient to whom insulin was administered.

Complications in diabetic patients were recognized very early. Japanese and Chinese of the third century A. D. observed a tendency by diabetics to furnunculosis (Papaspyrous 1952). By the fifth century A. D., furunculosis and tuberculosis were recognized in Europe as complications of diabetes.

Cheselden in the "Anatomy of the human body" (1750) was the first modern writer to note that diabetics were often plagued with boils and carbuncles. Before the introduction of insulin, pyogenic infections and tuberculosis were regarded as important diseases of diabetics. Tuberculosis at one time was responsible for the death of one-half of all those with diabetes (Joslin 1959). The diabetic patient is very susceptible to pulmonary tuberculosis. Figures given by Thiery (1933) indicated that this infection accounted for forty percent of all diabetic deaths. This percentage rising to sixty percent in severe diabetes accompanied by lack of assimilation of proteins.

Warren (1938) reported that acute infection was the cause of 197 deaths (37%) in 527 fatal cases of diabetes. Bronchial and lobar pneumonia were responsible for more deaths than any other localized infection. Urinary tract infections occur frequently in uncontrolled diabetes (Root and White 1956). The most common infectious agents are: Escherichia coli, Staphylococcus aureus and Staphylococcus albus, Proteus vulgaris, Streptococcus hemolyticus and Streptococcus faecalis, Aerobacter aerogenese, Pseudomonas aeruginosa and often mixed infections.

Persons with uncontrolled cases of diabetes are generally more susceptible to infection (Joslin et al 1959), since

with proper treatment the susceptibility of such patients to infection approaches the normal. Introduction of sulfanamides and antibiotics has contributed much to the protection of the diabetic against bacterial infection. However, they still present a condition predisposing to the development of fungal infections by members of the family Mucoraceae.

Gregory, Golden, and Haymaker (1943) reported mucormycosis involving the central nervous system in three diabetic patients. Le Compte and Meissner (1947); Bauer, et al., (1955); and Harris (1955) reported cases of diabetic patients with involvement of the central nervous system. Lloyd, Sexton, and Hertig (1949) described a case of pulmonary mucormycosis in a patient who showed features suggesting diabetes. The majority of these cases were fatal and generally the infecting organism was a species of the genus Rhizopus but fungal infection can be of any type. The sugar fermenting Candida (Buchanon 1962) finds an ideal medium in urine and tissues containing glucose. Optimal growth of yeasts and staphylococcis is found on media with 150-200 milligrams percent sugar, an amount which corresponds to the sugar content of serum of many diabetics. Diabetic vulvovaginitis due to yeast infection is encountered in about 50 percent of women with diabetes.

Experimental diabetes was first produced by pancreatectomy (Lukens 1948), and later by repeated injections of

anterior pituitary extract. More recently alloxan has been used for this purpose.

Jacobs (1937) reported that seventy or more mgms of alloxan per kgm. of body weight injected intravenously into rabbits produced initial hypoglycemia which led to convulsions and death of the animal within seven to ten hours, at which time the blood sugar concentration was less than 15 mgm%. Hypoglycemia and convulsions were relieved by intravenous injections of glucose. In some animals a transitory hyperglycemia preceded the severe fall in blood sugar concentration. Dunn, Sheehan, and McLetchie (1943) and Bailey and Bailey (1943) gave intravenous injections of alloxan to rabbits with a resulting initial hyperglycemia within the first hour followed by severe hypoglycemia which was generally fatal. Dunn et al. (1943) by studying the tissues of alloxan diabetic rats found complete necrosis of the islets of Langerhans while the acinar tissue escaped injury. Dunn and McLetchie (1943) were able to induce permanent diabetes in rats by intramuscular and subcutaneous injections of alloxan.

The diabetogenic effect of alloxan was soon confirmed by other workers. Golden and Gomori (1943) confirmed the observations of Jacobs, Dunn and co-workers in the rabbit and in addition extended their experiments to rats, guinea pigs, cats, dogs, and pigeons all of which were sensitive in

varying degrees to alloxan. These investigators were able to produce permanent diabetes in rabbits and dogs. Duffy (1945) obtained the same results in rabbits. Such observations on experimental alloxan diabetes in animal species offered an opportunity for research and investigation of susceptibility of such animals to infections.

Payne and Cruickshank (1948) made a comparison of antibody response to crystalline egg albumin in normal and diabetic rabbits. They found the diabetic state did not inhibit egg albumin antibody production.

Cruickshank and Payne (1949) reported that an encapsulated type II pneumococcus of low virulence inoculated into whole blood of alloxan diabetic rabbits incubated *in vitro* grew much more rapidly than when incubated under similar conditions in normal rabbit blood. The rate of growth in the former case was two or three times as rapid although initial bacterial counts were approximately equal. When these experiments were repeated using plasma instead of whole blood, growth was heavier in the normal plasma. Since no loss of phagocytic power in the leukocytes of diabetic animals was demonstrated, the authors concluded that the defect was possibly due to an inability of these phagocytes to destroy the ingested bacteria.

Cruickshank (1954) gave intravenous injections of virulent and avirulent pneumococci and virulent staphylococci

(coagulase positive S. aureus) to rabbits with chronic alloxan diabetes and to normal controls. A comparison of the rate at which the bacteria were removed from circulation, the survival rate of the animals and the reaction to lesions which developed in the two groups did not indicate impairment of resistance in the diabetic animals. Measurement of staphylococcal lesions in the skin of rabbits with chronic diabetes and normal controls failed to show any difference between the two groups. In addition, there was no difference in survival time of simular experimental and control animals injected with bovine tubercle bacilli nor in the extent of the lesions produced by this organism. Rabbits in acute toxic phase of alloxan diabetes associated with lipemia and ketosis showed marked failure of inflammatory response in the skin to staphylococcus injected intradermally. The author related this failure to peripheral circulatory collapse in the animals and not a consequence of the diabetic state.

Wertman and Henney (1962) showed increased susceptibility of alloxan diabetic rate to coagulase positive Staphylococcus aureus. When these animals were challenged with intraperitoneal injections of the organism, 100 percent of the diabetic rate developed bactermia with a fifty percent fatality rate. A non-diabetic control group simularly subjected to the organism produced no bactermia or fatality.

Phagocytic studies further indicated that the diabetic animals had impaired defense mechanisms. This was apparent in a lower percentage of active neutrophiles and in the diminished capacity of these neutrophiles to phagocytize Staphylococcus aureus.

Bauer, Flanegan, and Sheldon (1955) reported the Staphylococcus lesions produced experimentally by the intranasal instillation of spore suspensions of fungi of the order Mucorales in rabbits with alloxan induced diabetes closely resembled those of cerebral mucormycosis in man. Infection established in the nasal mucosa of hyperglycemic rabbits rapidly spread to adjacent tissues and disseminated to other parts and organs of the body. These animals showed a diminished inflammatory response. The lesions in the non-diabetic rabbits were few in number and of minute size not involving tissues beyond the submucosa.

Bauer, Flanagan, and Sheldon (1956) observed that rabbits inoculated with Rhizopus oryzae through intranasal instillation developed nasal, pulmonary, and cerebral mucormycosis even when the fungus inoculation preceded acute alloxan diabetes by several days. Rabbits with infusion hyperglycemia inoculated with the same organism developed fungus lesions in the nose and lungs. These lesions were more frequent and active than those of metabolically normal rabbits, but lacked the fulminating invasiveness found in

rabbits with acute alloxan diabetes. Since the blood sugar levels in both sets of hyperglycemic rabbits were generally the same, these workers suggested that high blood sugar concentration did not account for the difference of lesions in the two sets of animals but that metabolic changes in the alloxan diabetic animals appeared to effect the host. In both acute alloxan diabetes and infusion hyperglycemia, the inflammatory response in all lesions consisted chiefly of infiltration of numerous polymorphonuclear leukocytes with marked nuclear changes of pyknosis and karyorrhexis. These changes suggested that altered leukocytic function was an important factor in the pathogenesis of Rhizopus oryzae infection.

Sheldon and Bauer (1958) produced subcutaneous granulomata in rabbits by the injection of a spore suspension of Rhizopus oryzae. The fungus remained confined to the site of inoculation and ten weeks later could not be isolated by culture methods. The lesions eventually healed completely. Acute alloxan diabetes was produced in rabbits with subcutaneous granulomatous lesions of 8, 10, and 15 days duration and after varying periods of diabetic acidosis, autopsies were performed and the tissues studied by microscopic examination. The skin lesion section showed proliferation of the fungus with frequent invasion of adjacent tissues and blood vessels and frequently associated with early necrosis of the granuloma wall. Hyperglycemic rabbits without acute diabetes or

acetonuria had skin lesions not different from those of the controls. It appeared that activation of the infection occurred only in the presence of acidosis. The authors suggested that changes in host metabolism due to acute alloxan diabetes appeared to activate a quiescent infection.

Elder and Baker (1956) inoculated suspensions of spores of Rhizopus arrhizus intratracheally into rabbits in acute, toxic, and chronic (after 11 days of diabetes) phases of alloxan diabetes. Infection of rabbits in the chronic phase resulted in essentially no proliferation of the hyphae into the bronchi and lungs. Infection of rabbits in acute phase (ketosis and lipemia) caused ulcerative bronchitis and extensive pneumonia with vascular invasion and thrombosis resulting in eventual death of the animals within a few days. This condition in rabbits closely resembled fulminating pulmonary lesions of human mucormycosis which develop in uncontrolled diabetic and ketotic patients. These workers mentioned loss of phagocytic ability, ketosis, devitalization of tissues, and changes in leukocyte metabolism as possible factors in enhancing Rhizopus infection in acute phase of alloxan diabetes.

Schofield and Baker (1956) inoculated normal and chronically alloxan diabetic mice with Rhizopus intraperitoneally and intracerebrally and obtained results similar

to Elder and Baker. Tissue reactions in both normal and diabetic mice were similar.

Andrioile and Hasenclever (1962) suggested that alloxan by altering the relationship of host metabolism to infection provides more favorable growth conditions for yeast pathogens or renders the infected diabetic animal more susceptible. Mortality rate increased in alloxan diabetic mice inoculated with Candida albicans and C. tropicalis and death was readily produced in alloxan diabetic mice infected with normal and non lethal strains of C. guillermandi and C. parapsidosis also diabetic animals showed increased tissue susceptibility to C. albicans. In all cases, kidney tissue was found to be the only one in which progressive infection occurred.

STATEMENT OF PROBLEM

Wertman and Henney (1962) demonstrated that rats injected with alloxan developed hyperglycemia and were subsequently susceptible to bacterial disease. The purpose of this investigation was: (1) to determine the susceptibility of alloxan diabetic rate to the Lee strain of the influenza virus type B. (2) to study the immunologic response by the hyperglycemic rats to the viral agent. The necessary controls were included in both studies.

MATERIALS AND METHODS

Animals and Housing

Male albino rats of Sprague-Dawley strain were used throughout this investigation. All animals were housed in wide mesh screen cages with corresponding mesh bottoms. The animals were housed individually. A water bottle was provided for each cage and replenished twice a day for diabetic animals and once a day for non-diabetic animals. The animals were maintained on stock feed (Purina Laboratory Chow) of adequate amount.

Establishment of Diabetes

Alloxan in appropriate doses produces permanent diabetes in dogs, rabbits, and rats by causing necrosis of the islets of Langerhans, (Dunn, Sheehan, and McLetchie, 1943; Dunn and McLetchie, 1943; Bailey and Bailey 1943; and Golden and Gomori 1943,) and no serious damage to other tissues occurs.

A pilot study was conducted following the method of Lazarow and Palay (1946). It was found that the intravenous injections of forty mgms of alloxan per kilogram body weight produced elevated blood sugar concentration in a majority of the animals. A few required an additional injection to

obtain the desired blood sugar concentration. The failure to respond was possibly due to a faulty method of injecting.

Based upon this information, the animals for this study were divided into two groups, (1) animals which received no alloxan for normal controls and (2) animals which received 40 mg of alloxan per kilogram body weight.

The alloxan was dissolved in physiological saline and sterilized by Seitz filtration. The amount of alloxan to be administered was calculated and injected intravenously into the tail vein.

Method of Injection of Tail Vein

The rats were etherized and then placed in a cylindrical mailing carton of appropriate size. The carton was provided with air holes at one end and a single hole cut out at the other end through which the animal's tail protruded. With the rat inside the carton, its tail was held in a jar containing water at about 60°C for approximately one minute. The warm water made the tail vein more visible and after some practice the distal end could be easily entered with a number 25 or 26 hypodermic needle. (An elevation in blood sugar concentration indicated the establishment of diabetes.)

Determination of Blood Sugar Concentrations

Blood specimens were obtained by tail bleeding technique. The rats were first etherized and then restrained

in a cylindrical mailing carton as previously described. The carton was then fastened to a stand by means of two clamps. The protruding tail was then swabbed with alcohol and its tip out with a sharp pair of scissors. The flow of blood was encouraged by stroking the tail from its origin to the distal end. One to two mls, of blood were collected in a sterile tube, permitted to clot, ringed, and centrifuged within one hour. The serum specimens were removed aseptically using a Pasteur pipette and maintained at -20°C until blood sugar concentrations could be determined.

The serum to be tested was thawed, 0.1 ml removed using 0.2 ml pipettes and dispensed in 10 ml of tungstic acid solution for precipitation of protein according to Folin (1929). The tungstic acid serum mixture was allowed to react for ten minutes and then centrifuged. The supernatant was used for each sugar determination. The anthrone method (Umbreit, 1957) was employed for all determinations. The tubes were placed in boiling water bath for three minutes, after addition of the anthrone reagent to equalize temperature of the reaction, cooled and the color measured in the Coleman spectrophotometer at 650 mu wave length against a blank set at 100% transmission. The mg/100 ml. of blood sugar was determined by reference to a previously constructed standard curve (Hiller 1957).

A base line blood sugar concentration determination was obtained for each animal before the injection of alloxan. Further blood sugar concentrations were obtained at seven to ten days after injections of alloxan. A concentration of 75-150 mg per 100 ccs of blood was considered normal sugar level for the animals.

The Viral Agents

The viral agents were: (a) the Lee (Park Davis) strain of influenza type B obtained from the Department of Microbiology, University of Arizona. The virus had ten mouse lung passages and two chick embryo allantoic passages at Viral and Rickettsial Disease Laboratory, Berkeley, California. Previous passage history was not known. It was passed once more through the chick embryo at the University of Arizona. (b) The PR-8 (Squibb) strain of influenza type A obtained from the same source. The virus had eight chick embryos allantoic passages at Viral and Rickettsial Diseases Laboratory, Berkeley, California. Previous passage history was not known. It was passed once more through chick embryo at the University of Arizona.

Preliminary Aerosol Studies

At the onset of this investigation certain preliminary studies were necessary to determine the effect of viral aerosol on the experimental animals regarding their general

reactions, symptomatology, and infection. In the first of these studies, eight rats of the Sprague-Dawley strain having an average weight of 230 grams were divided into two groups of four each. The first group was exposed for five minutes to a dynamic aerosol (trial 1) sprayed from a solution containing a dilution of the PR-8 virus. The second group was exposed to aerosol sprayed from a solution containing a 1:10 dilution of the virus for the same amount of time and under same conditions.

The dynamic aerosol toroid (DAT), Goldberg et al. (1958) was primarily designed for the study of aged aerosol particles of up to six microns under controlled conditions of temperature, pressure, and humidity. It can also be used for transient flowing unaged aerosol. For this study, storage of aerosol in the drum was not required. A bypass arrangement permitted a direct flow of aerosol from the sprayer to the animal exposure compartment.

The frozen stock PR-8 test culture was thawed and diluted to the required concentrations in sterile (Difco) heart infusion broth (HIB) containing 100 units of Penicillin and Streptomycin (100 micro-grams) per ml. Two drops of Dow Corning Antifoam (Dow AF6) were added to the broth. The rats were placed individually in separate compartments of a cage 48" x 6" x 6" containing twenty compartments and connected to the aerosol toroid outlet. Aerosol was generated

from a modified Collison atomizer (Henderson 1952) containing the spray sample. Samples were collected in two all glass impingers (AGI-30) (Sampling Microbiological Aerosol 1959) containing 22.5 mls of the same broth diluent used in the spray suspension.

Impingers were operated during the five minute spray period and a five minute subsequent air wash with clean filtered air. The modified Collison atomizer was operated at 28 pounds pressure per square inch. Total air flow was 95.8 liters per minute at 76°F and 50% relative humidity.

At the end of the ten minute exposure period, the animals were returned to their cages and impinger and spray samples frozen in duplicate for assay later.

The exposed animals were observed for eight days for any abnormal signs, such as watery nasal discharge or diminished food or water intake which would indicate symptoms of infection. During this period, no such symptoms were observed. Two rats, one from each group were then chloroformed in a large glass jar and autopsied using aseptic technique. Careful examination of their lungs suggested no abnormalities of any kind.

A group of ten rats, including the remaining six from above, were employed in an attempt to adapt the influenza virus to the animals by the method of serial passage (Harris 1937). Three rats in this group were first made

diabetic by intravenous injection of 40 mg. of alloxan per kgm. body weight. Because some of the rats had been previously exposed to PR-8, the Lee strain of influenza type B was used since it is heterologous to antibodies specific for the PR-8 strain which may have been present in the animals previously exposed.

The procedure was initiated by exposing three rats to an aerosol sprayed from a 1:3 dilution of the virus (trial 2) for 30 minutes in the manner as previously described. Frozen virus culture was thawed and diluted in HIB containing antibiotic and antifoam. During the first five minutes of aerosol generation, an aerosol sample was obtained with one AGI-30 containing 22.5 ml of HIB, antibiotic and antifoam. The modified Collison atomizer was operated at 28 pounds pressure per square inch. Total air flow after sampling was 70.8 liters per minute at 76°F and 50% relative humidity.

The rats were killed on the third day and their lungs removed aseptically, weighed, ground with sterile sand and emulsified in heart infusion broth. The suspension was made up to a concentration of 25% and centrifuged lightly. Five drops of the supernatant were inoculated intranasally into each of two rats anaesthetized with ether. The remainder of the suspension was frozen for assay at a later date. Subsequent passages were carried out on the second, or third day in an identical manner. The procedure was continued for

five passages. The lung suspensions from the first and second passages were each inoculated into groups of three mice using the same amount and technic as for the rats, (Table III). The mice were killed on the sixth day and their lungs examined for lesions.

None of the rats showed external symptoms. The lungs from the rats of the third and fifth passages had certain areas of discoloration and one or two minute foci which appeared to be possible lesions. These were very doubtful and in appearance were not considered as indications of influenza infection.

Embryonated chicken eggs were inoculated with lung emulsions from rats used in the investigation (Table III), and based on the data as obtained from this study, adaptation of the virus to the rats was abandoned at this stage.

A further study was conducted to determine whether the virus was capable of multiplying in the lung tissue of the rats. Ten five week old rats were exposed for 30 minutes (trial 3) to an aerosol sprayed from a dilution of the influenza B virus, Lee strain in the DAT in the manner previously described. During the first five minutes of aerosol generation, aerosol samples were taken with two AGI-30 impingers. Total air flow after sampling was 64.5 liters per minute at 76°F and 50% relative humidity. Aliquots of the spray suspension and pooled impinger fluids were frozen in duplicate for later assay.

Two rats (A and B) were killed every twenty four hours after exposure for the following five days and their lungs carefully examined for signs of lesions, after which the lungs were removed, weighed, ground and a 10% suspension prepared as previously described. The suspensions were frozen for assay later in embryonated eggs.

Embryonated Egg Inoculations

Embryonated chicken eggs were employed to determine whether viral multiplication occurred within the lung tissues of the animals. The lung suspensions were removed from deep freeze and thawed at room temperature. Ten fold dilutions of each lung suspension were made resulting in three dilutions ranging from 10^{-1} to 10^{-3}.

After completing the dilution sequence, for all lung suspensions, 0.1 ml quantities of each dilution were inoculated into the allantoic sac of eleven day old chick embryos. The method of egg inoculation and viral harvest followed that prescribed by the expert committee on Respiratory Virus Diseases, The World Health Organization (1959) and Manual of Laboratory Methods in Virology (1963).

The method of testing embryos for virus replication essentially followed a principle presented by Burnet and Beveridge (1943). The serologic tubes were arranged in ascending order for each separate dilution with eight tubes

per dilution. A 0.25 ml of a 1% chicken erythrocyte suspension was added to each tube. Several tubes were included as controls containing only 0.5 ml of saline and red blood cells. The tubes were shaken and incubated at room temperature for approximately one hour by which time the controls had settled into a "button" deposit. Tubes showing hemagglutination were recorded as positive for presence of virus.

Susceptibility and Immunologic Studies

In the main part of this investigation, 34 alloxan diabetic and 33 non diabetic male albino rats of the Sprague-Dawley strain twelve weeks old weighing 250 grams on the average were employed. All animals were exposed to an aerosol (trial 4) sprayed from a suspension of undiluted stock virus. Four successive exposure trials were necessary in order to subject all the animals to the aerosol. Identical conditions of temperature, pressure, and humidity were maintained during all four exposures. Freshly thawed stock (undiluted) influenza virus type B, Lee strain was used as the spray suspension, to which antibiotics and antifoam were added as in previous trials. Each rat was placed in an individual compartment of exposure unit cage just prior to aerosolization and exposed to the spray suspension for 30 minutes. Sample of each aerosol was taken with one impinger.

During the first five minutes of the spray period, total air flow after sampling was 70.8 liters per minute

throughout the aerosolization with the following temperatures and relative humidity recorded for each respective exposure:

1st	76°F	48-49%	relative humidity
2nd	76°F	48-49%	relative humidity
3rd	74.8°F	48-49%	relative humidity
4th	74.5°F	50-51%	relative humidity

Aliquots of the spray suspension and impinger fluids for each exposure were frozen for subsequent assay.

After aerosol exposure, the animals were divided at random into three main groups of exposed diabetic and non diabetic rats. Each main group was then divided into two sub groups, one of which was comprised of experimental diabetic, exposed rats (sub group a) and the other of non diabetic exposed controls (sub group b).

Animals in groups one and two were employed in susceptibility studies and those of group three were studied for immunologic response to the viral infection. All animals were observed for the first twelve hours to rule out death due to non specific causes.

Based on previous pilot studies, all animals in groups one and two were sacrificed on the third and seventh day respectively after exposure to the aerosol. Animals in group three were held for twenty-one days after exposure to permit the production of antibodies. At the end of this period,

they were bled from the tail for serum samples. After bleeding, the animals in this group were sacrificed.

To avoid exerting any strain on the animal's lungs by chloroform vapor, they were sacrificed by a blow to the base of the neck in the area of atlas and axis vertebrae with a heavy blunt instrument. The lungs were carefully exposed using aseptic procedures and examined for signs of lesions and/or any other macroscopic abnormalities. The following arbitrary scheme was used for quantitative scoring of lung lesions:

-; normal

±; trace of consolidation in one or more lobes

1+; Consolidation of significant portions of one lobe or multiple minute foci in two or more lobes.

2+; Consolidation of major portions of one lobe or significant portions of two or more lobes.

3+; Consolidation of one lobe complete or major portions of two or more lobes.

4+ Consolidation of one lobe complete plus major portions of other lobes.

5+ All lobes consolidated

After gross examination, the lungs of the rats in groups one and two were removed, washed in sterile physiological saline and stored in the frozen state in a separate, appropriately labelled petri dish. Later each lung was thawed, weighed and ground aseptically in a mortar and

pestle with sufficient sterile saline to comprise a ten percent suspension. The addition of a small amount of sterile sand facilitated the grinding procedure. The suspensions were then incubated at 37°C. for twenty minutes to encourage any virus to elute from the homogenized debris and then centrefuged at 2,000 revolutions per minute for ten minutes. The supernatant fluid was withdrawn and stored at -60°C. in screw capped tubes.

Tray Method for Viral Assay

This method essentially followed the technique developed by Fazekas De St Groth and White (1958) for assaying influenza viruses.

A standard medium suggested Fazekas De St Groth and White (1958) was used throughout the experiment for viral assay. Eleven day old eggs found to be most suitable regarding susceptibility and physical condition of the membrane (D. O. White and Fazekas De St Groth 1959) were de-embryonated and rinsed three times with approximately 5 ml. volumes of standard medium. The shell containing the chorioallantoic membrane was cut into 6 x 6 mm. squares and stored in a Petri dish containing standard medium. One square of membrane on shell was transferred into each cup of prepared trays* containing 0.3 ml. volumes of the standard medium. A serial ten fold dilutions of a certain number of the

*W. H. O model 8x10 plastic cup tray

suspensions were made comprising dilutions 10^{0} to 10^{-4}. All dilutions were made with standard medium and maintained in an ice bath until the trays were inoculated.

Eight cups were inoculated and each cup received 0.05 ml. of the test material. Batteries of four trays were stacked on one another separated by spacers and covered by a blank on top to allow sufficient air space. The sides of each battery was surrounded by moistened cheese cloth, then wrapped and sealed with plastic film to prevent desiccation. Pairs of batteries were mounted on a horizontal shaker of 120 oscillations per minute and incubated at 96°F for 72 hours. Three days was found to give maximum titer for influenza virus type B. (Fazekas De St Groth and White 1958). After incubation, the trays were removed, the pieces of egg shell and membrane were picked out with fine forceps and a standard drop (0.025 ml.) of 10% chicken red blood cells added to each cup. The trays were then shaken thoroughly and allowed to stand 30 minutes at room temperature before recording the results. All assays were performed in duplicate.

Lung suspensions, representing seventeen animals of group one and two which exhibited varying degrees of gross lesions of the lung, were assayed for content of virus. In a majority of cases, hemagglutination readings were negative or indicated very low virus titers. To ascertain the presence of any virus, a similar group of lung suspensions as above

was assayed using the same technique as employed above, however, sixty cups were inoculated with 0.05 ml. quantities of the undiluted suspension. A number of these suspensions were assayed by both procedures.

The viral assays strongly indicated loss of virus in the lung suspension of the experimental animals; therefore, a comparative study of viral replication in standard medium and lung suspension was undertaken to evaluate the validity of this observation.

Lung suspension from rat number 32 was chosen for this purpose since it had no macroscopic lung pathology and zero titer when tested by the tray method and, therefore, assumed to be a normal organ. Five sterile tubes were prepared as diluent blanks with 2 ml. of heart infusion broth in the first and 1.8 ml. in the other blanks. Aseptic procedures were employed throughout. Stock influenza B, Lee was thawed and 0.925 ml. transferred to the first blank, mixed and 0.2 ml. delivered to the second blank. A clean sterile pipette was used to transfer 0.2 ml. from the second to the third blank and the ten fold serial dilutions continued to the fifth blank. This procedure resulted in a final dilution of $10^{-4.5}$. Eight new blanks were prepared, half of which contained 2 ml. quantities of heart infusion broth and the other half 2 ml. quantities of the lung suspension. A 0.925 ml. aliquot of the $10^{-4.5}$ dilution of the

virus was transferred to the first tube of each set. Serial dilution was continued carrying 0.925 ml. quantities of each mixed dilution to the next tube, thus, obtaining three half-log dilutions ranging from $10^{-5.5}$ to $10^{-6.5}$. These dilutions were titrated in 0.05 ml. aliquates using tray assay method as previously described. Each dilution was inoculated into ten cups.

Rats in group three were involved in determination of their immunologic response to the viral infection.

Three weeks after aerosolization, the animals in this group were bled and sacrificed as previously described. The blood was allowed to coagulate at room temperature and then centrifuged at 1,200 r.p.m. for ten minutes. The clear serum was removed with a Pasteur pipette and stored at -20°C. in an appropriately labelled tube. Later the serum was thawed and inactivated at 56°C. for thirty minutes before determining the antibodytiter.

Hemagglutination Tests

The tests described here and under hemagglutination inhibition (HAI) follow essentially that prescribed by the World Health Organization (1959) and Manual of Laboratory Methods in Virology (1963).

A series of ten two fold dilutions in saline of influenza virus type B, Lee strain was prepared in test tubes

(14-78 mm.) in the following manner:

1. 0.8 ml. of physiologic saline was put in tube #1 and 0.5 ml. in each of other tubes (2-11)

2. 0.2 ml. of stock virus was added to tube #1, mixed with a clear pipette and 0.5 ml. transferred to tube #2.

3. This serial transfer was continued thru tube #10. 0.5 ml. was discarded from tube 10.

4. The dilutions thus prepared, ranged from 1:5 thru 1:2560.

5. One additional tube (#11) containing only 0.5 ml. saline served as the control.

6. 0.5 ml. of 0.5% chicken red blood cells was added to each tube, shaken well and allowed to stand at room temperature (22-25°C.) and the titer read when the control formed a "button" deposit on the bottom of the tube, usually within thirty minutes.

From the hemagglutination titer, the virus dilution containing four hemagglutination units (HAU) per 0.25 ml. was calculated and used as test antigen in the hemagglutination inhibition (HAI) test. The highest dilution which completely agglutinated the standard erythrocyte suspension was one HAU, that contained one unit of HA activity. For example, in this particular case the HA titer was 1:640, the working dilution desired for the HAI test is four units per 0.25 ml. therefore 640/16, or a dilution of 1:40.

Hemagglutnation Inhibition Test:

Working dilution for the HAI test was prepared containing four HA units of virus per 0.25 ml. A 1:10 dilution

of each heat inactivitated serum was made.

1. Ten serial two-fold dilutions of each antiserum was made in saline using 0.25 ml. quantities of antiserum.

2. 0.25 ml. of virus dilution containing four HA units was added to each tube and mixed by shaking. All tubes were allowed to stand at room temperature for fifteen minutes.

3. 0.5 ml. of 0.5% chicken red blood cells were added to each tube and shaken well.

4. The tests were allowed to stand at room temperature and hemaglutinations read in thirty minutes.

An antigen control was run concurrently with the HAI test to substantiate that only four HA units of antigen were added to each tube of the test.

1. Five serial two-fold dilutions of the virus were made in saline using 0.5 ml. quantities of the antigen used in the HAI test (1:40).

2. 0.5 ml. quantities of 0.5% chicken red blood cells were added to each tube, shaken well and left at room temperature for thirty minutes. Hemagglutinations were read at the end of this period.

RESULTS

Throughout this investigation, experimental conditions were maintained as similar as possible in order to avoid variations in results and to allow a methodical representation of results obtained.

In the first aerosol trial (trial 1) eight rate of Sprague-Dawley strain were divided into two groups of four each. One rat in each group was made diabetic by intravenous injection of 40 mg./kgm. of alloxan as described.

The first group (A) was exposed for five minutes to a dynamic aerosol sample sprayed from a 1:1000 dilution of PR-8 influenza virus and the second group (B) to an aerosol sprayed from a 1:10 dilution of the virus for the same period of time. This was followed in both instances by five minutes of subsequent air wash with clean filtered air. Table 1 represents spray and impinger sample assays of the virus. The fifty percent end point, EID_{50} per ml. for the respective spray and impinger samples in trial 1, were calculated by the method of Reid and Muench (1938) and are presented in the same table. An estimation of respiratory dose of virus received by each rat was calculated in the following manner:

Group A:

Virus titer of impinger contents 3.73×10^{1}/1.0 ml.

Volume of impinger contents 22.5 ml.

Total impinger collection $(22.5)\ (3.73 \times 10^{1})$

$= 8.4 \times 10^{2} EID_{50}$

Sampling rate of impinger $= 12.5 \times 10^{3}$ cc/min.

Average weight of animal = 250 gms.

Respiratory rate of rat $= (2.1)\ (250)^{3/4}$ cc's/min.

(Guyton, 1947) = 133cc/min.

The animals and samplers were exposed to the aerosol for the same length of time, therefore, the respiratory dose of the animal is that proportion of the total impinger collection as determined by the ratio of the sampling rate of the impinger to the respiration rate of the animal:

$$\text{ratio} = \frac{\text{respiratory rate of rat}}{\text{aspiration of impinger}}$$

$$= \frac{133 \text{ cc/min.}}{12.5 \times 10^{3} \text{cc/min.}} = 10.6 \times 10^{-3}$$

respiratory dose 10^{3}cc/min. $= (10.6 \times 10^{-3})\ (8.4 \times 10^{2})$

$= 8.9\ EID_{50}$

Group B:

Virus titer of impinger contents $= 2.4 \times 10^{3}\ EID_{50}$

Since conditions under which animals were exposed were the same for animals in Group B as in Group A, the respiratory

dose = (22.5) (2.4×10^3) x (10.6×10^{-3})

= 572 EID_{50}

All animals exposed to the PR-8 aerosol in trial 1 were observed during the following eight days for symptoms of infection, such as watery nasal discharge or diminished food and water intake; however, no such signs were apparent. On the ninth day, one rat from Group A and one from Group B were sacrificed -- their lungs were exposed and examined for lesions or other pathological signs. Careful observations of these organs suggested no abnormalities of any kind.

The remaining rats from the above Groups A and B plus a group of unexposed animals were then used in an attempt to adapt the influenza virus to these animals by serial passage Harris (1937). Since some of the rats had been exposed to PR-8 and had possibly developed a specific antibody titer to the virus, the Lee strain of influenza virus type B was used (trial 2) as the test organism. The experimental procedure has been described. Table II gives spray and impinger sample assay of this trial as determined by tray assay method as well as respective fifty percent end point EID_{50}/ml. Estimation of the respiratory dose received by each rat was calculated according to the following:

Virus titer of impinger contents = $3.98 \times 10^4 EID_{50}$ per ml.

Sampling rate of impinger = 12.5×10^3 cc's/min.

Volume of impinger contents = 22.5 cc's

Average weight of animals = 250 gms.

Time of exposure = 30 min.

Time impinger sample collected = 5 min.

Respiratory volume = 133 cc's/min.

Therefore EID_{50}/ml. of aerosol = $\frac{3.8 \times 10^4 \times 22.5}{12 \times 10^3 \times 5}$

Total volume respired by animal = respiratory volume x time of exposure = 133 x 30

Therefore dose per animal = $\frac{3.98 \times 10^4 \times 22.5 \times 133 \times 30}{12.5 \times 10^3 \times 5}$

$= 5.7 \times 10^4 EID_{50}$'s

None of the animals in this trial showed signs of infection and inoculations into mice and egg embryos of lung suspensions of rats after the second passage indicated that no virus multiplication was occurring in the lungs of the rats at the second passage. Table III summarizes the results of mouse and egg inoculations of the influenza virus passed serially in these rats.

So far in this study external symptoms and superficial lung tissue examinations indicated the virus to be incapable of infecting the rats and an attempt at adaptation of the virus to the animals was unsuccessful. This seemed to be equally true for alloxan diabetic or normal animals; therefore, a further experiment was initiated to determine whether any

viral growth or multiplication occurred in the lung tissue of the rats, notwithstanding the absence of lung pathology.

Ten five week old rats of 80 grams average weight were involved in this study as previously described in aerosol trial three. Aerosol exposure time, aerosol concentration, and conditions of temperature and humidity were the same as in trial two. Two animals (A and B) were sacrificed every twenty-four hours after exposure for the following five days, their lung examined for lesions or other abnormalities and a ten percent suspension prepared as previously described. These suspensions were assayed in embryonated eggs under the standard method described and their fifty percent end point (EID_{50}) determined. These results are represented in Table IV.

These results indicated that the virus was being maintained in the lung tissue of the animals and appeared to be growing actively on days four and five after an initial drop in titer on days two and three. Viral titer appeared to be lowest on day three and highest on day five. It was postulated that this titer may be high enough by day seven to cause lung pathology; therefore, it was decided to test for susceptibility of the normal alloxan diabetic rats to the viral infection three and seven days after exposure and to determine if animals in diabetic state showed increased susceptibility as compared to normal controls. To encourage

pathology in the lungs undiluted stock influenza virus B was used as a spray suspension to achieve a substantial increase as previously described in the aerosol concentration of the virus (Trial 4). Table V represents spray and impinger sample assays, and fifty percent end points as determined by tray method.

Diabetes was established in thirty-four animals as previously discussed and a further thirty-three were used as normal controls. Blood sugar concentrations of 75-150mgs. per 100 cc's. were considered normal, higher values indicating hyperglycemic state. Table VI represents distribution and blood sugar concentration of rats in mgs./100 cc's. of blood.

All rats were observed daily during the period following exposure for indications of infection; however, no physical symptoms were observed. Rats in groups one, two, and three were sacrificed on the third, seventh and twenty-first day respectively as previously described and their lungs examined for gross pathology. The results of these observations are presented in Table VII which gives qualitative scores of all lesions under the arbitrary scheme already discussed.

A certain percent of animals in each group showed lung lesions of varying degree as follows:

Rats in group 1 sub group A (diabetic) 58.3%
Rats in group 1 sub group B (non-diabetic) 36.3%
Rats in group 2 sub group A (diabetic) 36.3%
Rats in group 2 sub group B (non-diabetic) 54.5%
Rats in group 3 sub group A (diabetic) 27.4%
Rats in group 3 sub group B (non-diabetic) 63.6%

These results indicate that lesions were produced in both diabetic and non-diabetic animals of all three groups in a random manner and independent of the physiological state of the animals pointing to the fact that alloxan-hyperglycemic rats are not more susceptible to influenza type B virus than normal healthy rats.

A certain number of lung suspensions from group 1 and 2 were assayed in duplicate (a and b) by the tray method as previously described. Table VIII presents the data on those assayed.

A similar group of lung suspensions were assayed by inoculating sixty cups with undiluted suspension in an attempt to detect very low titers of virus which might pass undetected when using fewer replicates for each dilution.

The data in Table IX represents the results of these assays.

The results of the tray assays, suggesting that lung titers of influenza virus were either negative or very low

was contrary to what was expected on the basis of the preliminary studies in which lung titers were confirmed by inoculation into mice and egg embryos. This contradiction in the data suggested that perhaps the lung tissue suspension was not a suitable medium for membrane-on-shell technique of assaying the virus and possibly inhibited viral multiplication. A comparison of influenza virus titation by the tray method using standard medium (control) and lung tissue suspension was therefore conducted. As the results in Table X indicate, rat lung tissue suspension seem to be unsuitable for and inhibitory to growth of the virus as assayed by the membrane on shell techniques, this is confirmed by the fact that viral growth occurs when dilutions are prepared in normal lung tissue and assayed in embryanated eggs, Table Xa.

Results of Immunological Studies

This section of the study involved twenty-one rats of group three and was concerned with determination of their immunologic response to the viral infection. This group contained hyperglycemic and normal controls. The serum from these animals was tested for their hemogglutination inhibition titer as previously described. Tables XI presents the results of hemagglutinations test.

The working dilution used = $\frac{640}{16} = 40$; a 1:40 dilution of virus was used. This is equal to four units of hemogglutinating (HA) activity. A concurrent dose control was run

with the hemagglutination inhibition (HAI) test to determine if, in fact, four units of HA activity were used. Table XII presents the results of dose control and Table XIII those of the hemagglutination inhibition test.

TABLE I

CONCENTRATION OF PR-8 INFLUENZA VIRUS IN SPRAY SUSPENSIONS AND IMPINGER SAMPLES (TRIAL I) AS DETERMINED BY INOCULATION IN EMBRYONATED EGGS

	Dilution	No. of Eggs Inoculated	No. Infected	No. Not Infected	EID_{50}/ml
Spray Suspension Group A	$10^{-3.0}$	10	10	0	$10^{5.62}$
	$10^{-3.5}$	10	10	0	
	$10^{-4.0}$	10	9	1	
	$10^{-4.5}$	10	6	4	
	$10^{-5.0}$	10	2	8	
Spray Sample Group B	$10^{-5.0}$	10	10	0	$10^{7.39}$
	$10^{-5.5}$	10	9	1	
	$10^{-6.0}$	10	9	1	
	$10^{-6.5}$	10	4	6	
	$10^{-7.0}$	10	1	9	
Impinger Suspension Group A	10^{-0}	10	8	0‡	$10^{1.57}$
	$10^{-0.5}$	10	7	3	
	$10^{-1.0}$	10	5	5	
	$10^{-1.5}$	10	2	8	
Impinger Sample Group B	$10^{-2.0}$	10	5	5	$10^{3.38}$
	$10^{-2.5}$	10	3	7	
	$10^{-3.0}$	10	1	9	
	$10^{-3.5}$	10	1	9	
	$10^{-4.0}$	10	0	10	

‡ In all cases the difference between these readings and total number eggs inoculated indicates non-specific "death" of egg embryos.

TABLE II

CONCENTRATION OF TYPE B LEE INFLUENZA VIRUS IN SPRAY SUSPENSIONS AND IMPINGER SAMPLES (TRIAL 2) AS DETERMINED BY THE TRAY METHOD

	Dilution	No. of Cups Inoculated	No. Infected	No. Not Infected	EID_{50}/ml
Spray Suspension	10^{-2}	20	20	0	$10^{5.02}$
	10^{-3}	20	18	2	
	10^{-4}	20	6	14	
	10^{-5}	20	2	18	
	10^{-6}	20	0	20	
Impinger Sample	10^{-1}	10	9	0	$10^{4.86}$
	10^{-2}	10	6	3‡	
	10^{-3}	10	8	2	
	10^{-4}	10	5	5	

‡ In all cases the difference between these readings and total numbers of cups inoculated indicates non-specific "death" of egg embryos.

TABLE III

PERSISTENCE OF INFLUENZA B LEE VIRUS IN RAT LUNG DURING SERIAL PASSAGE AS DETERMINED BY INTRA-NASAL INOCULATIONS IN MICE AND INOCULATIONS INTO EMBRYONATED EGGS

Rat Nos.	Passage	Inoculum	Day of Sacrifice	Rat Lung Lesions	Presence of Viruses Judged by Mouse Inoculation of Rat Lung Suspension	EID_{50}/ml
1,2,5+	0	Aerosol Influenza B Lee	3	0	+++	$10^{2.4}$
4,9+	1st	Lung Suspension	2	0	±	0=
6	2nd	"	2	0	*	0 *
8	3rd	"	2	0	*	0
11+,12	4th	"	3	0	*	0
13	5th	"	5	0	*	0

* Mouse inoculations were not performed.

= No viral infection of inoculated eggs.

+ These animals were diabetic.

TABLE IV

CONCENTRATION OF INFLUENZA B LEE VIRUS (TRIAL 3) IN LUNGS OF PAIRS OF RATS AS DETERMINED BY INOCULATION INTO EMBRYONATED EGGS, FOLLOWING INFECTION BY THE AEROSOL ROUTE

Day of Sacrifice	Dilution of Lung Suspension	No. of Eggs Inoculated	A Infected	A Not infected	A EID_{50}/ml	B Infected	B Not infected	B EID_{50}/ml	Aver. EID_{50}/ml	Lung Lesions in Rats
1	10^{-1}	8	7	0‡	$10^{3.0}$+	8	0	$10^{3.0}$	$10^{3.0}$	None
	10^{-2}	8	8	0		6	0			
	10^{-3}	8	8	0		6	2			
2	10^{-1}	10	8	1	$10^{2.5}$	8	1	$10^{2.75}$	$10^{2.63}$	None
	10^{-2}	10	6	2		7	2			
	10^{-3}	10	3	6		2	6			
3	10^{-1}	8	7	1	$10^{2.0}$	5	0	$10^{1.75}$	$10^{1.85}$	None
	10^{-2}	8	3	4		1	5			
	10^{-3}	8	2	5		1	7			
4	10^{-1}	8	7	1	$10^{2.42}$	None		None	$10^{2.42}$	None
	10^{-2}	8	7	1						
	10^{-3}	8	1	7						
5	10^{-1}	8	8	0	$10^{2.57}$	8	0	None	$10^{2.57}$	None
	10^{-2}	8	7	0		7	0			
	10^{-3}	8	1	7		6	2			

+ Dilutions did not go far enough to give an end point, at 10^{3} all eggs were infected.

‡ The difference between these readings and total number of eggs inoculated indicates number of dead eggs before harvesting.

TABLE V

CONCENTRATIONS OF INFLUENZA B LEE VIRUS (TRIAL 4) IN SAMPLES FROM SPRAY SUSPENSION AND IMPINGER CONTENTS AS DETERMINED BY THE TRAY METHOD FOLLOWING EXPOSURE OF RATS TO AEROSOL.

Sample	Virus Dilutions	No. of Cups Inoculated	No. Injected	No. Not Injected	MID_{50}/ml
Spray	10^{-3}	10	7	3	$10^{4.8}$
	10^{-4}	10	3	7	
	10^{-5}	10	0	10	
	10^{-6}	10	0	10	
Impinger 1	10^{-1}	10	4	6	
	10^{-2}	10	2	8	$10^{2.3}$
	10^{-3}	10	0	10	
Impinger 2	10^{-1}	10	9	1	
	10^{-2}	10	3	7	$10^{2.95}$
	10^{-3}	10	0	10	
Impinger 3	10^{-0}	10	10	0	
	10^{-1}	10	10	0	$10^{3.14}$*
	10^{-2}	10	2	8	
	10^{-3}	10	0	10	
Impinger 4	10^{-0}	10	9	1	
	10^{-1}	10	9	1	$10^{2.96}$
	10^{-2}	10	3	7	
	10^{-3}	10	1	9	

* MID_{50} in this case was obtained by the linear mortality grid of Goldberg, (1954).

TABLE VI

RANGE AND AVERAGE VALUES OF BLOOD SUGAR CONCENTRATIONS OF RATS

Group	Sub-Group	Amt. Alloxan/kilogram Body Weight mgm	Total No. Rats	Initial Conc. mg sugar/100 cc of blood aver.	Initial Conc. mg sugar/100 cc of blood range	Final Conc. mg sugar/100 cc of blood aver.	Final Conc. mg sugar/100 cc of blood range
1	A	40	12	99	75-150	345	160-675
	B	0	11	108	75-150	116	75-150
2	A	40	11	109	75-150	333	160-500
	B	0	11	122	75-150	111	75-150
3	A	40	11	123.2	75-150	283.5	178-430
	B	0	11	111	85-150	115	85-150

TABLE VII

QUALITATIVE SCORE OF LUNG LESIONS OF RATS EXPOSED TO INFLUENZA B LEE VIRUS (TRIAL 4) ACCORDING TO DAY AFTER AEROSOL EXPOSURE

Group I - Third Day

Rat No.	*Diabetic	Rat No.	*Non-Diabetic
1	2+	40	3+
2	4+	44	-
5	2+	46	2+
6	-	49	-
7	3+	53	-
8	1+	56	-
9	-	59	2+
10	-	64	-
28	-	66	-
32	-	68	1+
37	2+	72	-
39	4+		

* Sub group A in text.

\+ Sub group B in text.

TABLE VII-Continued

QUALITATIVE SCORE OF LUNG LESIONS OF RATS EXPOSED TO INFLUENZA B LEE VIRUS (TRIAL 4) ACCORDING TO DAY AFTER AEROSOL EXPOSURE

Group II - Seventh Day

Rat No.	*Diabetic	Rat No.	+Non-Diabetic
11	1+	42	4+
14	-	45	-
16	-	48	-
18	-	51	-
19	±	54	±
21	-	58	4+
23	1+	61	-
26	1+	63	2+
29	-	65	1+
33	-	67	2+
36	-	70	-

* Sub group A in text.

\+ Sub group B in text.

TABLE VII-Continued

QUALITATIVE SCORE OF LUNG LESIONS OF RATS EXPOSED TO INFLUENZA B LEE VIRUS (TRIAL 4) ACCORDING TO DAY AFTER AEROSOL EXPOSURE

Group III - Twenty-First Day

Rat No.	*Diabetic	Rat No.	+Non-Diabetic
12	-	41	-
13	±	43	1+
15	-	47	-
20	±	50	1+
22	-	52	-
24	-	55	±
25	-	57	±
27	±	60	1+
30	-	62	±
31	-	69	±
34	-	71	-

* Sub group A in text.

\+ Sub group B in text.

TABLE VIII

PRESENCE OF INFLUENZA VIRUS, B LEE STRAIN, IN LUNGS OF DIABETIC AND NORMAL RATS FOLLOWING AEROSOL EXPOSURE TO THE VIRUS (TRIAL 4) **

Animal No.	Day of Sacrifice	Diabetic	Lung Lesion	Dilusion of Lung Suspension	No. of Cups Inoculated	A Infected	A Not Infected	B Infected	B Not Infected
2	3	+	4+	10^{-0}	8	1	7	0	8
				10^{-1}	8	0	8	0	8
				10^{-2}	8	0	8	0	8
				10^{-3}	8	1	7	0	8
				10^{-4}	8	0	8	0	8
7	3	+	3+	10^{-0}	8	2	6	1	7
				10^{-1}	8	1	7	0	8
				10^{-2}	8	1	7	0	8
				10^{-3}	8	0	8	0	8
				10^{-4}	8	0	8	1	7
32	3	+	-	10^{-0}	8	0	8	0	8
				10^{-1}	8	0	8	0	8
				10^{-2}	8	0	8	0	8
				10^{-3}	8	0	8	0	8
				10^{-4}	8	0	8	0	8
40	3	-	4+	10^{-0}	8	0	8	0	8
				10^{-1}	8	0	8	0	8
				10^{-2}	8	0	8	0	8
				10^{-3}	8	0	8	0	8
				10^{-4}	8	0	8	0	8

TABLE VIII-Continued

PRESENCE OF INFLUENZA VIRUS, B LEE STRAIN, IN LUNGS OF DIABETIC AND NORMAL RATS FOLLOWING AEROSOL EXPOSURE TO THE VIRUS (TRIAL 4) **

Animal No.	Day of Sacrifice	Diabetic	Lung Lesion	Dilusion of Lung Suspension	No. of Cups Inoculated	A Infected	A Not Infected	B Infected	B Not Infected
64	3	-	-	10^{-0}	8	0	8*	0	8*
				10^{-1}	8	0	8	0	8
				10^{-2}	8	0	8	0	8
				10^{-3}	8	0	8	0	8
				10^{-4}	8	0	8	0	8

* Eleven other diabetic and non-diabetic rats in group 1 and 2 showing various degrees of lesions and similarly assayed, had identical readings.

** Virus assays conducted with the allantois-on-shell technique (tray method).

TABLE IX

LUNG TISSUE ASSAY OF INFLUENZA B VIRUS (TRIAL 4) IN DIABETIC AND NORMAL RATS AS DETERMINED BY TRAY METHOD USING UNDILUTED SUSPENSION, FOLLOWING EXPOSURE TO AEROSOL

Animal No.	Day of Sacrifice	Diabetic	Lung Lesions	No. of Cups Inoculated	No. Infected	No. Not Infected
40	3	-	3+	60	0	60
53	3	-	-	60	0	60
32	3	+	-	60	0	60
33	7	+	-	60	0	60
44	3	-	-	60	0	60
63	7	-	2+	60	0	60
7	3	+	3+	60	11	49
48	7	-	-	60	4*	56
45	7	-	-	80	0	80
70	7	-	-	60	0	60
21	7	+	-	60	0	60
58	7	-	4+	60	0	60
36	7	+	-	60	0	60

* A further 36 cups showed what appeared to be partial hemagglutination.

TABLE X

COMPARISON OF TITERS OF INFLUENZA B LEE VIRUS USING STANDARD MEDIUM AND NORMAL RAT LUNG SUSPENSION AS DILUENTS, AS DETERMINED BY THE TRAY METHOD

Medium Used	Viral Dilution	No. of Cups Inoculated	No. Infected	No. Not Infected	MID_{50}/ml
Standard	10^{-5}	10	8	2	$10^{6.08}$
	$10^{-5.5}$	10	7	3	
	$10^{-6.0}$	10	5	5	
	$10^{-6.5}$	10	4	6	
Normal Rat Lung Suspension	10^{-5}	10	0	10	No Infection
	$10^{-5.5}$	10	0	10	
	$10^{-6.0}$	10	0	10	
	$10^{-6.5}$	10	0	10	

TABLE Xa

TITERS OF INFLUENZA B LEE USING NORMAL RAT LUNG SUSPENSION AS DILUENT, AS DETERMINED BY EMBRYONATED EGG METHOD *

Viral Dilution	No. of Eggs Inoculated	No. Infected	No. Not Infected	EID_{50}/ml
10^{-7}	9	8	1	
10^{-8}	9	3	6	$10^{8.2}$
10^{-9}	9	2	7	

* The influenza B Lee Virus was of a different stock, however had close titer to the original.

TABLE XI

PROTOCOL AND RESULTS OF ANTIGEN TITRATION FOR HEMAGGLUTINATION-INHIBITION TEST

Tube No.	1	2	3	4	5	6	7	8	9	10
Viral Dilution (Final)	20^{X}*	40^{X}	80^{X}	160^{X}	320^{X}	640^{X}	1280^{X}	2560^{X}	5120^{X}	$10,240^{X}$
Hemagglutination	+	+	+	+	+	+	-	-	-	-
HA Units	32	16	8	4	2	1	1	1	1	1

* This indicates a dilution of 1:20, 1:40 and so on.

TABLE XII

PROTOCOL AND RESULTS OF DOSE CONTROL TEST FOR HEMAGGLUTINATION-INHIBITION TEST

Tube	1	2	3	4	5
Viral Dilution (Final)	160^{X*}	320^{X}	640^{X}	1280^{X}	2560^{X}
HA	+	+	+	-	-
HA Units	4	2	1	1	1

The results of the actual hemagglutination inhibitions test are given in table XIII.

* This indicates a dilution of 1:160 and so on.

TABLE XIII

CONCENTRATIONS OF INFLUENZA HEMAGGLUTINATION INHIBITING ANTIBODY IN SERA OF NORMAL AND DIABETIC RATS 21 DAYS AFTER AEROSOL EXPOSURE TO INFLUENZA B LEE VIRUS

Virus Tubes		1	2	3	4	5	6	7	8	9	10
		Final Serum Dilution									
Animal No.	Diabetic	40^X	80^X	160^X	320^X	640^X	1280^X	2560^X	5120^X	10240^X	20480^X
		Hemagglutination Inhibition Titer									
12	+	+	+	+	+	+	+	+	-	-	-
13	+	+	+	+	+	+	+	+	-	-	-
15	+	+	+	+	+	+	-	-	-	-	-
20	+	+	+	+	+	+	+	+	-	-	-
22	+	+	+	+	+	+	+	+	+	-	-
24	+	+	+	+	+	+	+	+	+	-	-
25	+	+	+	+	+	+	+	+	-	-	-
27	+	+	+	+	+	+	-	-	-	-	-
30	+	+	+	+	+	+	+	+	-	-	-
31	+	+	+	+	+	+	+	-	-	-	-
34	+	+	+	+	+	+	+	-	-	-	-
43	-	+	+	+	+	+	+	-	-	-	-
47	-	+	+	+	+	+	+	+	-	-	-
50	-	+	+	+	+	+	+	+	-	-	-
52	-	+	+	+	+	+	+	+	+	-	-
56	-	+	+	+	+	+	+	-	-	-	-
57	-	+	+	+	+	+	-	-	-	-	-
60	-	+	+	+	+	+	+	-	-	-	-
62	-	+	+	+	+	+	+	-	-	-	-
69	-	+	+	+	+	+	-	-	-	-	-
71	-	+	+	+	+	+	-	-	-	-	-

DISCUSSION

It was apparent from this study that, alloxan diabetic rats and non-diabetic controls respond in the same manner to infection with the Lee strain of influenza B virus. In the susceptibility studies this was indicated by lack of any significant difference in infection, antibody production, symptoms, or lung lesions. The latter point is indicated by the fact that:

Rats in group 1, sub group A (diabetic) had 58.3% lung lesions.

Rats in group 1, sub group B (non-diabetic) had 36.3% lung lesions.

Rats in group 2, sub group A (diabetic) had 36.3% lung lesions.

Rats in group 2, sub group B (non-diabetic) had 54.5% lung lesions.

The percent lung lesion in the diabetic groups were not significantly different from non-diabetic control groups. In respect to the ability of the diabetic and non-diabetic animals to produce antibody, again no significant difference was noticed as indicated by the hemagglutination inhibition titer of the diabetic and control groups (Table XIII); in all cases, a titer of at least 1:640 was obtained by all animals in diabetic and non-diabetic control groups.

Payne and Cruickshank (1948) reported that the diabetic state did not inhibit antibody production to crystalline egg albumin in rabbit. If the results of the present study in rats can be accepted as further extension of their observations it would suggest that cellular and humoral mechanisms involved in susceptibility and resistance to viral infection are equally manifested in the experimental and control animals. The virus, as a foreign antigenic particle, naturally ellicits antibody production in its host and in this respect the present study confirms such defense mechanism in diabetic and normal animals and further indicates infection of the animals in both groups. At the level of virus host cell interaction, however, certain observations have been made which merit further discussion. Preliminary studies indicated multiplication of virus in the lung tissue of the experimental animals as shown by the results (trial 3) represented in Table IV. This group of animals had no symptoms of infection or gross pathology. The initial drop in the titer on days two and three which is followed by an increase on days four and five probably represents an intercycle phase between the first and second cycles of infection during which, the particles were probably released from primarily infected cells. This interim period between day one and day four being used in a sequence of absorption and entry into the new cells, followed by multiplication, maturation and liberation of the newly infective virus particles.

Based on this observation it was speculated that a more concentrated initial viral aerosol could cause a more severe infection and possibly more pronounced pathology in the lung tissues, therefore, in the main part of this investigation (trial 4), undiluted stock influenza virus type B was used. As seen in Table VII a certain percent of animals in both diabetic and normal group, did produce lung lesions of various degree, which as mentioned above was not significantly different.

The results in Table X and Xa indicate that the egg membrane-on-shell technique is an unsuitable method for viral assay using rat lung suspension as diluent and that embryonated eggs are preferable, obviously the chick embryo as a susceptable host greatly enhances viral replication and growth as compared to the lung suspension from an artificial host. The host-parasite interaction certainly differs widely in the laboratory animals and eggs as supported by the following points:

1. Toxic effect: With a high enough concentration of virus, cells may be damaged (Burnet 1960), but insufficient virus produced to allow infection of further cells of the same type. This effect is typically demonstrated by certain viruses in the influenza group on intracereberal inoculation of mice and by Newcastle disease virus administered intranasally. In the latter case Burnet (1942) demonstrated

that the virus, in high concentrations causes extensive consolidation of the lungs. Subsequent passage experiments, however, failed completely and very little virus was found to be present in the consolidated lungs. Anderson and Burnet (1947) noted that when an unadapted strain of influenza A virus was transferred to mice using undiluted allantoic fluid virus, the mice first inoculated showed marked lung lesions, however, these lesions did not occur on subsequent passages. The characteristics of these lesions were the same as those produced by much smaller amounts of adapted virus.

2. Incomplete virus: This concept of incomplete virus as demonstrated by influenza viruses is predominatly due to Von Magnus (1951, 1952). In serial passage of undiluted allantoic fluid Von Magnus observed a dissociation between infectivity and hemagglutinating ability of the virus. The ratio of former to latter decreased with increased transfer or passage of the undiluted virus. It appears that in the chick embryo at least, sufficiently large initial inoculum infects all cells of the allantois in the first cycle and leads to production of very large proportion of "incomplete" virus. Such response is not observed in case of small inocula when two or three cycles of liberation and reinfection occurs. The "incomplete" virus is a laboratory artifact obtained in an artificial host with unusually large incula.

3. Action of host cells. A further factor in virus cell interaction is the nature of the host cell and its action on the invading virus. It is plausible to assume that such virus develops new characteristics as dictated by host conditions. Further, action of natural inhibitors; such factors (Grossberg 1962) as species, sex, nutritional state, hormonal balance, enzymes age and genetic characters may exert great influence upon host susceptibility and resistance. Such factors seem to have had the same effects in alloxan diabetic and normal rats and, therefore, indicate that the diabetic state does not interfere to any great extent with the resistance or susceptibility to infection with influenza virus and the immunologic response of the animals studied.

SUMMARY

Male albino rats of Sprague-Dawley strain were injected with alloxan to determine effect of diabetes on (1) susceptibility of these animals to the Lee strain of influenza virus type B, (2) to study the immunologic response by the hyperglycemic rats to the viral agent. The necessary controls were included in both studies.

The following results were obtained: The diabetic state did not seem to have any effect on the susceptibility of the animals to the influenza B Lee virus. This was demonstrated by lack of any difference in infection, symptoms or lung pathology.

The ability of the alloxan diabetic and non-diabetic rats to produce antibody was not significantly different as indicated by the hemagglutination inhibition test. A high titer of at least 1:640 was obtained by all animals in the two groups.

An attempt to adapt the virus to the strain of rats used was unsuccessful and the animals were found to be highly resistant to the B, Lee strain of influenza virus administered via an aerosol.

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