EPIDEMIOLOGICAL STUDIES OF INFLUENZA

by

Julius Abraham Nii Addo Mingle, B.Pharm., Dip. Bact.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada.

AUGUST, 1969

© Julius Abraham Nii Addo Mingle 1969

ACKNOWLEDGEMENTS

The author wishes to thank Dr. J.C.N. Westwood, Professor and Head of Department, for his immense help and interest during this work and who in spite of his many duties found time to advise and direct this project. My thanks also go to Mr. Peter Phipps, Laboratory Scientist, Virus Laboratory, Civic Hospital, who acted as the technological adviser. I extend my sincere gratitude to the following: Dr. M.A. Klotz and Dr. R.D. Strickland, Ottawa Civic Hospital, for allowing us the use of the blood samples, without which this work would not have been possible.

I wish also to thank Dr. E. Perry, Miss Charlotte Rigby and Dr. R.K. Chaudhary for their advice and suggestions during the preparation of the manuscript; Mr. L. Guerin for providing me with some valuable information and Mr. Anthony Brunner, for drawing the figures and for his warm companionship and help throughout the course of this project, and Miss Emelia G. Adom for typing this work.

I am also grateful to the Canadian International Development Agency who sponsored my studies.

TABLE OF CONTENTS

Page

Introduction 1

Literature Survey

1. History of Pandemics 3

2. Antigenic Variation of Influenza Viruses 10

   (i) Man as Source of New Variants 11

   (ii) Animals as Reservoirs 14

   (iii) Recombination of Influenza A Viruses of human and Animal Origin 17

3. Factors affecting the Epidemiology of Influenza 18

   (i) Weather 18

   (ii) Crowding 19

   (iii) Age of the Population 20

   (iv) Others 21

4. Antibody Pattern and its Relationship to Immunity 23

   (i) Pattern of Antibody Formation 23

   (ii) Antibody Types 24

   (iii) Types of Complement Fixing Antibodies 25

   (iv) Effect of Influenza A infection on Antibodies to Influenza B 26

   (v) Immunity and Antibody level 27

Page

5. Population Survey of Influenza 30

(i) Laboratory Diagnosis 30

(ii) Serum Survey 32

(a) Predictive Survey 33

(b) Retrospective Survey 34

Part I - Serologic Survey of Influenza Antibodies

Materials and Methods 36

(i) Sera 36

(ii) Diluent 36

(iii) Haemolysin 37

(iv) Sheep red blood cells 38

(v) Antigens 39

(vi) Complement 40

Complement Fixation Test 41

Results 45

Discussion 78

Part II - Persistence and Immunoglobulin nature of anti "S" and anti "V" CF Antibodies

Introduction 85

Materials and Methods 85

Results 87

Discussion 91

Page

5. Population Survey of Influenza 30

(i) Laboratory Diagnosis 30

(ii) Serum Survey 32

(a) Predictive Survey 33

(b) Retrospective Survey 34

Part I - Serologic Survey of Influenza Antibodies

Materials and Methods 36

(i) Sera 36

(ii) Diluent 36

(iii) Haemolysin 37

(iv) Sheep red blood cells 38

(v) Antigens 39

(vi) Complement 40

Complement Fixation Test 41

Results 45

Discussion 78

Part II - Persistence and Immunoglobulin nature of anti "S" and anti "V" CF Antibodies

Introduction 85

Materials and Methods 85

Results 87

Discussion 91

Page

General Summary 95

Appendix.............................. 96

References 101

INTRODUCTION

The effect of absenteeism on the economy as a result of influenza epidemic, not to mention excess in mortality, cannot be over-emphasized. Rhodes and Van Rooyen (1962) reported that during the epidemic of 1918, deaths due to influenza alone were more than the number of people killed in World War I.

In view of the global character of influenza and the sudden nature of its onset and spread, much interest has been stimulated to study the periodicity and pattern of epidemics and if possible to forecast future epidemics so that adequate steps would be taken to reduce the severity of influenza.

Zhdanov (1959) remarked that the only effective way of minimizing the morbidity as a result of influenza epidemic is through immunization.

Fukumi (1959) observed from his studies on human vaccination experiments with the Asian 'flu in Japan, that the tempo of an epidemic can be altered if immunization is done close to the epidemic season. Dempster (1968) remarked that vaccines are most effective when given a few weeks before an epidemic.

These observations bring into focus the need for

means of predicting an outbreak of influenza.

Zhdanov (1967) used serologic surveys to predict two epidemics in 1965 and 1967 in the USSR.

Pereira et al (1967) remarked that the susceptibility of a community to current strains of influenza virus is an important factor in the development of epidemics of influenza. Serologic survey of the human population for antibodies against the current strains of the virus gives an indication of the degree of susceptible people in that community since people with demonstrable antibodies are partially protected and usually run a mild course of infection.

The present work was therefore undertaken

(1) To assess the antibody level in the population of Ottawa as an index of susceptibility to the current strains of influenza viruses.

(2) To follow the expected incidence of influenza during the period of the study and to determine what type of influenza was involved.

(3) Attempts were also made to study the persistence of complement fixing antibodies against the "soluble" and "viral" antigens of influenza A and the immunoglobulin nature of these antibodies.

LITERATURE SURVEY

1. HISTORY OF PANDEMICS

Influenza had been recognized and described as an acute respiratory disease commonly occurring in epidemic form, long before the advent of virology.

Literature contains many descriptions of such epidemics, covering many areas of the world over many centuries.

Francis and Maassab (1965) mentioned an epidemic described by Short which occurred in most parts of the world in 1557. They observed that from 1510 to 1930 some 30 outbreaks considered as pandemics occurred.

Rhodes and Van Rooyen (1962) remarked however that the first real pandemic was recorded in 1847 to 1848, and observed that during the first half of the 19th Century there were a few widespread outbreaks of influenza. The disease apparently disappeared from most parts of the world for nearly 40 years.

In 1887 some influenza was recorded in Russia and in 1889 during the summer in Central Asia (Rhodes and Van Rooyen 1962). Later in that year the disease

spread to other parts of Russia and to Western Europe and this started the 1889-91 pandemic.

Cantlie (1891) described an epidemic of febrile respiratory disease in 1888 in Britain.

Wilson and Miles (1965) reported that the 1889-92 pandemic was followed by intermittent epidemics which culminated in the great pandemic of 1918-19.

This pandemic is recorded in the literature as the most lethal in history and they summarized the severity of the pandemic thus: "This was one of the worst plagues of human history... destroying more lives in a few months than did the Great War in 4 years". It is believed that the disease killed between 10 - 20 million persons.

The 1918 pandemic was recorded as having begun in the summer almost simultaneously around Boston and Brest in the United States. (Andrewes 1967). These were ports for embarkation and disembarkation for American troops to and from Europe and it was believed that the virus might have been seeded into the troops during their European combats.

The severity of this pandemic served as a stimulus to discover the causal agent. It was not however until 1933 when Smith, Andrewes and Laidlaw reported the recovery of a filtrable virus from a case of influenza in London. At the same time Francis reported the isolation of a virus from cases in Puerto Rico and New York.

In 1940, Magill and Francis independently isolated a new type of influenza virus during an outbreak in New York. Influenza viruses have since then been found to exist in three immunologic types, A, B, and C (Rhodes and Van Rooyen 1962).

Influenza A causes widespread epidemics and pandemics and Influenza B is usually involved in localized outbreaks with occasional worldwide outbreaks (Robinson 1964). Influenza C infects mostly children.

Accounts of earlier pandemics strongly suggest that influenza A viruses were involved in those pandemics (Andrewes 1967).

In 1946-47 it was observed that a new antigenic variant of influenza A was circulating in the world population and this caused yet another pandemic.

The 1946 pandemic was recorded to have started from Korea and Japan where the first outbreaks were noticed. Later an outbreak occurred in the United States. This strain was widely prevalent for at least 10 years.

In 1947, the WHO set up the World Influenza Information Centres in many countries of the world charged with responsibility of surveilling changes and epidemics of influenza.

In 1957 a new A virus appeared again on the world scene to cause another pandemic. This pandemic of influenza was the first that it has been possible to study using modern virological techniques in an almost world-wide network (Payne 1958). The first outbreaks occurred in the hinterland of China; however, the precise point of origin is not known (Jensen et al 1958 quoting Tang and Liang).

Dunn (1958) reported that the disease was first reported in late February 1957 in China. From established foci in countries along the eastern fringe of Asia, the disease spread to many new areas in May.

The virus earned its name the Asian 'flu due to its point of origin.

Rhodes and Van Rooyen (1968) reported that by April 1957, the disease had spread throughout the territory of China to south east Asia and Australia and from these points it went all over the globe.

It was ironical that the epidemic should originate in an area not covered by the WHO programme. Had it been that China was a participating country in this programme, the rest of the world might have had an additional two months in which to take appropriate measures for control.

The initial wave of the pandemic touched almost every country and major territory before November, 1957 (Robinson 1964). In September, 1957 a second wave occurred in many countries that had it in April and in some cases the new wave was more severe, especially in those countries where the first wave had been mild. An influenza was reported in the U.S. in January 1959 and in April of the same year in Canada.

Robinson (1964) wrote that the 1959 outbreak failed to reach epidemic proportions in most areas. North America experienced another outbreak from January to March in 1960 with an unusual severity

causing excess mortality closely approaching that of the fall of 1957. The eastern coast of Canada was the most affected during this epidemic. Further incidences have occurred with significant increase in 1962.

The situation remained almost quiet until late summer/autumn of 1968 when a new variant of influenza was noticed in Hong Kong. This new strain has since been the cause of new waves of epidemic in many countries. North America was affected with the new strain before the Christmas of 1968 and many states in the U.S. reported high percentages of the disease in the New Year (Prier et al 1969).

In Canada most of the outbreaks were centres in and around Montreal with some cases in Ottawa.

Moderate epidemics of influenza B occurred in 1946 and 1959 in most parts of England (Stuart-Harris 1965) and a more extensive outbreak occurred during 1961 and 1962.

First global appearance of influenza B was started in Czechoslovakia in December 1958 and then in Canada around February 1959 (Robinson 1964). From that time onward, type B had made its appearances in many countries with an outbreak in Canada in

November, 1961. This outbreak was suspected to have been introduced from the Carribean area and Alaska where it had been noticed earlier on. From that time up to the present there have been few cases of influenza B in many parts of the world and these are being followed with interest since it is not perhaps impossible that an epidemic with B can replace the usually A pandemics.

2. ANTIGENIC VARIATION OF INFLUENZA VIRUSES

A basis for more scientific and precise knowledge of influenza and its etiology was laid in 1933 when Smith et al isolated the first influenza virus. Since then, many antigenic variants of the virus have been isolated and studied extensively. (Table 1).

The original strain isolated in London by Smith et al and in the U.S. by Francis are known as the Influenza A-classic (Ao) (Rhodes and Van Rooyen 1962). This type prevailed until 1946.

In 1946 a new strain with different antigenic structure appeared. This was designated as A-prime (A1). This new antigenic type prevailed until 1957 when the A2 or the Asian 'flu was isolated.

There has been a progressive change also in the antigenic structure of Influenza B virus (Rhodes and Van Rooyen 1968). Strains isolated recently can be distinguished from the Lee strain which is the prototype of the first isolate, although recognition of subtypes in influenza B is not achieved as in Influenza A.

As yet little is known about influenza virus C, but what is known points to a considerable degree of

stability in the antigenic structure. (Zhdanov 1959). Strains of influenza C isolated in 1947 in the U.S. are identical to those isolated in 1957 in the USSR. There is no record of any changes thus far in Influenza C viruses.

The fact that pandemics are associated with the emergence of antigenically novel viruses is a well known phenomenon (Rhodes and Van Rooyen 1968). Studies have been undertaken to investigate the origin of such "antigenic drift" thereby providing effective way of coping with new strains by vaccination.

Three different theories have been advanced to explain the appearance of such new strains:

(a) that the source of new antigens is probably man himself.

(b) the appearance of new viruses without evidence of continuous mutation from prior human strains suggests that they might have come from animal reservoirs.

(c) that new variants have emerged as a result of recombination of Influenza A viruses of human and animal origin (Kilbourne 1968).

(i) Man as Source of New Variants

WHO Expert Committee (1959) suggested that the source of interpandemic outbreaks in man is probably

TABLE I

INFLUENZA VIRUS TYPE A (HUMAN) AND TYPE B MAJOR MUTANTS*

GROUP	NAME OF MUTANT FORM OF VIRUS	PROTOTYPE	YEARS OF PREVALENCE
A	Influenza A human (Classic or Ao)	PR 8	1933-1946
	Influenza A human (A prime or A1)	FM 1	1947-1957
	Influenza A human (A Asian or A2)	Japan 305	1957 to date
	?Influenza A human (A3)	Hong Kong	1968
B	Influenza B (Lee)	Lee	1940-1948
	Influenza B (Great Lakes)	GL	1950 to date
	Influenza B Japan/7/56	Japan/7/56	1956 to date

* Modified from Textbook of Virology - Rhodes and Van Rooyen (1968).

? Satz et al (1969).

man himself and that the antigenic variations happen under the "driving force of selection" due to the immune status of the population.

Magill (1955) performed an experiment in which he demonstrated that influenza viruses can survive and propagate in "immune" environments induced in mice by vaccination with the homologous strain of the virus. Survival of the virus was associated with the emergence of variants which differed from the parent strain in antigenic characteristics. He remarked that perhaps mutation had occurred and mutants had been selected by the immune status of the mice used, and suggested that a similar process might be happening in the human population.

In 1959, Zhdanov put forward a thesis explaining the immunological features of influenza and its relationship to the emergence of new variants. He observed that there is a transition from a condition of immunity to infection to a condition of immunity to disease after influenza infection. The virus is able to multiply during the later part of this immunological state and the variation in the level of immunity of the population serves as a mechanism of selection of new antigenic variants.

There is however, yet another explanation of antigenic variations. Antibodies to the influenza A2 were demonstrated in older people before the 1957 outbreak (Mulder and Masurel 1957). They remarked that the Asian 'flu variant might have circulated earlier in the world or that it was antigenically similar to the strains which were prevalent during the childhood of these people. Davenport and Hennessy (1958) remarked that the presence of such antibodies might be due to the fact that there is recycling of antigens of influenza and that old strains reappear again in subsequent pandemics.

Isaacs et al (1962) reported that one strain of influenza virus A1 was recovered from a soldier in England at a time when A1 seemed to have disappeared completely with the first appearance of A2 in 1957. They presented evidence to rule out this isolation as being a laboratory pick-up and suggested that either the virus has been circulating unobserved since 1955; the time of the last reported isolation of virus of that antigenic character, or that the soldier was a carrier.

(ii) Animal as Reservoirs or Sources

A report by Shope (1931) describing a strain of influenza A virus isolated from cases of febrile illness

in swine, clarified the etiology of a disease prevalent in these animals at that time. In 1938 he reported that the swine influenza virus was the surviving prototype of the virus causing the 1918 pandemic which affected both swine and human, and suggested that the disease in swine originated from human infection. It has since been speculated that swine or other animals might be sources for new variants (Wallace and Kissling 1959). Since Shope's swine virus, animal influenza viruses have been isolated from horses, fowls and ducks.

In 1957, the World Health Organization arranged a survey of horse and swine sera in order to gain information on the role and importance of animals in the epidemiology of influenza. (Kaplan and Payne 1959). From the results, it was apparent that the Asian strain can cause natural inapparent infection in horses and swine.

Recently new influenza A viruses have been isolated from several avian species. (Pereira et al 1965). Pereira et al (1967) showed that one of the isolated strains is antigenically related to human influenza A2.

Couch et al (1969) examined the serological relationship between influenza A/equi 2 and the Hong Kong strains of human influenza A2. They infected antibody-free volunteers with influenza A/equi 2. These volunteers developed febrile upper respiratory illnesses which in most cases were indistinguishable from naturally-occurring influenza. Their results suggested that equines may play a role in emergence of new influenza strains that will infect susceptible human populations. Johnson and Westwood (1967) reported the presence of influenza A/PR8 precipitins in the serum of some normal animals namely rabbits, guinea pig, roosters, a goat and a sheep. Further work by these authors (1969) indicated that 6 out of 12 normal rabbits examined later had precipitins which demonstrates the extent of the presence of influenza antibodies in these normal animals. Fyson and Westwood (1969) also reported the presence of precipitins to various influenza strains of human and animal origins in normal rabbit sera. These results suggest the possibility of a wider range of myxovirus infection in nature and possible involvement of animals in epidemics than had been demonstrated.

(iii) Recombination of Influenza A viruses of Human and Animal Origin

Genetic interaction between human A2 virus and viruses of animal origin has been observed in chick embryo cell cultures (Tumuva and Pereira 1965). Kilbourne (1968) reported the production in the laboratory of stable hybrids arising as a result of genetic recombination of influenza A/equine and human A2. Simpson (1969) wrote that perhaps there was much to be gained from such studies and that this may give more information for effective control of influenza.

Burnet, Hirst, Fraser and others have also done similar studies extensively (Rhodes and Van Rooyen 1968).

3. FACTORS AFFECTING THE EPIDEMIOLOGY OF INFLUENZA

(i) Weather: History indicates that epidemics or pandemics of influenza are most likely to occur during the winter months (Andrewes 1967).

Zhdanov (1959) reported that climate and season affect the spread of influenza. From his studies in the USSR during the 1957 pandemic, he observed that the first wave of infection which occurred in the summer, had a low morbidity; during the second wave in October, morbidity was much higher. He did further studies to establish the direct effect of seasonal factors on the spread of influenza. A group of participants at the World Youth Festival in the USSR which took place during the summer of 1957 were studied. There were fewer cases of influenza among the participants at that time than in a group from the general population. He indicated that contact between the members of this summer group was as close as could be obtained during the winter in the general population and yet still the outbreak was milder during the summer. He concluded that seasonal factors do directly influence the spread of influenza, and

suggested that this may be due to the verucidal effect of sunlight.

Jensen et al (1957) suggested that season has been the most important factor determining the occurrence of epidemics due to the influenza A2. During May/August, 1957, the pandemic was clearly restricted to the South Hemisphere where winter was in progress. European countries and North America were not affected until the fall. Andrewes (1967) wrote that there was evidence of introduction of the A2 virus into England during the summer months of 1957 but it did not spread. It was only when the cooler autumnal weather came along that the outbreaks started. Hemmes et al (1960) studied the influence of temperature and humidity on the survival of influenza viruses. They noticed that the viruses when suspended in aerosol form survived better under conditions of temperatures and humidity similar to those obtained during the Winter months in the temperate zone. It is however puzzling as to how the viruses manage to spread without any difficulty in the tropics (Andrewes 1967)

(ii) Crowding: Influenza is one of the examples of a droplet-spread respiratory infection and as such dissemination of the viruses is increased in over-crowded

or close communities (Rhodes and Van Rooyen 1968). There is an increase in morbidity during influenza outbreaks among soldiers in camps and children in boarding schools as well as other institutions as compared to the general population (Robinson 1964).

(iii) Age of the Population: The spread of influenza is conditioned by the increase in the number of susceptible persons among the population, in the form of new-born and growing children (Zhdanov 1959). Morbidity due to influenza A is highest in children and the younger age group. (Rhodes and Van Rooyen 1968). A considerable percentage of new-born children receive passive immunity from their mothers both during their intra-uterine development and the breast-feeding (Zhdanov 1959). For this reason, children up to six months old usually have very low susceptibility to influenza. In addition, at that age they are less exposed to infection, since they have less contact with other children and adults. Children between 2 and 3 years of age are more susceptible to influenza (Zhdanov 1959) because at that age they have lost their passive maternal immunity. The course of primary infection is therefore more severe than it is in adults who have

residual immunity produced by a previous attack.

Morbidity due to influenza C occurs chiefly in childhood because infection with Influenza C affects mostly children (Zhdanov 1959), and since strains of Influenza C have not changed very much, there is a high level of herd immunity in the population which is maintained by repeated subclinical infection in older children and adults.

Mortality due to influenza A is highest in young children and old people (Rhodes and Van Rooyen 1968). Infection in childhood is primary in nature which is more severe than re-infection as seen in young adults (Zhdanov 1959). Ritova (1959) remarked that there is more marked toxicosis and more frequent involvement of the lungs in primary infection.

In older people mortality is increased due to complications as a result of secondary bacterial infection which may precipitate heart and other chronic diseases.

(iv) Others: The spread of influenza is affected by improved lines of communication since these help in the dissemination of the virus from one point of the world far more easily than previously. Many

outbreaks have occurred earlier in certain communities than anticipated, as a result of arrivals from "epidemic" regions of apparently healthy carriers (Rhodes and Van Rooyen 1968). Robinson (1964) suggested that rapid spread of influenza viruses during the 1957 outbreak may have been due to massive introduction of the infection into countries through ports, railway networks etc.

Socio-economic factors also play a part in the rapid spread of influenza viruses since social habits and economic standards are known to affect the dissemination of droplet-spread diseases.

4. ANTIBODY PATTERN AND ITS RELATIONSHIP TO IMMUNITY

(i) Pattern of Antibody Formation

Francis (1953) and Davenport et al (1953) suggested that influenza virus attacking young infants or children for the first time imprints its antigen upon the antibody forming mechanism and this determines the response to reinfection later on in life. This thesis has come to be known as the phenomenon of "original antigenic sin". Recall responses in individuals are at their highest to the strains which cause childhood infection (Davenport et al 1953). Infection with a new strain of influenza virus causes the formation of antibodies, not only against the offending strain, but also against strains of previous infections. Antibodies therefore reflect prior experience with the virus according to antigenic family and type (Hilleman et al 1958). With increasing age and repeated infection by antigenic variants antibody is formed against a greater number of antigens of influenza viruses. This explains why young adults are better protected against infection than infants (Hilleman et al 1958; Davenport and Minuse 1964).

(ii) Antibody Types:

Three types of detectable antibodies are formed after infection with influenza namely:

i) Virus neutralizing antibodies

ii) Haemagglutination inhibition antibodies (HI)

iii) Complement fixing antibodies (CF).

After infection, neutralizing antibodies appear first, followed by haemagglutination inhibition antibodies and then by complement fixing antibodies. Antibodies appear in the blood a few days after infection and reach peak levels in about 10 days (Jawetz et al 1962). The duration for which peak levels are maintained however has been a question of much speculation. Neutralizing antibodies are maintained at peak levels for a longer time than HI antibodies and usually CF antibodies decline to undetectable levels within a few months (Rhodes and Van Rooyen 1968). Hobson et al (1957) observed in a vaccinated group of 50 people that the haemagglutination inhibition antibody peak titres to influenza A2 occurred 1 month after vaccination and in 68% of this group these levels were maintained for one year. Jawetz et al (1962) wrote that peak levels are maintained for about 5 weeks after infection and they

declined gradually to pre-infection levels within a year. Francis and Maassab (1965) stated that vaccine-induced antibodies may persist at high levels for several years after vaccination, especially when a secondary response has been induced. Pereira et al (personal communication 1968) reported that the three types of antibodies to influenza A were still detectable over a period of 11 years in a group of 25 families they studied.

(iii) Types of Complement Fixing Antibodies

Influenza complement fixing antibodies are of two types:

a) anti-soluble (anti S) antibodies, which are formed against the viral nucleoprotein. (Lief and Henle 1959). These antibodies are type-specific; i.e. they distinguish influenza A, B or C. They have no protective significance. (Rhodes and Van Rooyen 1968).

b) Anti-viral (anti V) antibodies which are strain specific and are closely related to the haemagglutination inhibition antibodies. Anti V antibodies are produced against the virus mucoprotein and lipoprotein antigens and

they play a major part in immunity (Rhodes and Van Rooyen 1968).

Both anti S and anti V antibodies are produced after infection or immunization with live virus. Only anti V is produced after immunization with killed virus; if anti S is produced, the titre is usually less than 1 in 8 (Davenport and Minuse 1964; Schmidt and Lennete 1965).

Fazekas de St. Groth and Donnelley (1950a) studied the antibody response to influenza A in mice, and observed that the production of complement-fixing anti S antibody is characteristic of infection. Other workers have reported similar observations (Kirber and Henle 1950; Hobson and Pearson 1965). Lief and Henle (1959) observed that anti S response in children is usually poor and that in adults anti S antibodies appear before anti V. Normally anti V titres are higher than anti S titres, and anti S declines more rapidly than anti V (Schmidt and Lennette 1965). Recall is expressed in both anti S and anti V antibodies (Hobson and Pearson 1965).

(iv) Effect of Influenza A infection on antibodies to Influenza B

It is generally believed that infection with influenza A has no effect on antibody to influenza B (Rhodes and Van Rooyen 1968).

Widelock et al (1959) however reported that infection with influenza A can affect the antibody titre to influenza B. They noticed during an outbreak in New York City that although influenza A infection was confirmed by laboratory isolations, there was a concurrent rise in antibody titres to influenza B in the population. Prezsmycki et al (1959) reported a similar observation when studying the response to influenza vaccine in Poland. They reported an increase in post-vaccination antibodies against the immunizing strain of influenza A, and a smaller increase against influenza B, which was not contained in the vaccine. Influenza B infection had not been reported during the period of the survey.

v) Immunity and Antibody levels

Influenza attacks selectively the mucosal cells of the upper respiratory tract without first invading the blood. Antibodies must be present in sufficient concentration at the site of infection of the virus to have any effect on the course of disease. This can only be achieved if antibody level in the blood is high (Rhodes and Van Rooyen 1968). Circulating antibodies therefore have only an indirect

relationship to protection (Fazekas de St. Groth and Donnelley 1950 a). Fazeka de St. Groth and Donnelley (1950b) noticed in a group of mice they studied that those with high pre-challenge antibody titres were protected better than those with low titres. Fukumi (1959) studied the relationship between antibody level and natural reinfection in man. He reported that reinfection occurred when preinfection antibody titres were mostly 1 in 16 or 1 in 32 as determined by the haemagglutination inhibition technique; only in very few cases did reinfection occur when preinfection titre was 1 in 64. He concluded that those who have haemagglutination inhibition titre more than 1 in 64 are very largely protected from reinfection.

After influenza infection, acquired immunity may last from 2 to 3 years (Pickles et al 1947); Slepushkin 1959) or longer. (Dempster 1968. Repeated attacks can be explained by the antigenic variability of influenza viruses i.e. the emergence of new variants. (Rhodes and Van Rooyen 1968).

Francis et al (1944) however, reported a study in which human volunteers infected artificially with influenza B were challenged four months later with

the same virus and 30% fell sick. The severity of disease was, however, reduced. Similar results were reported by Henle et al (1946) using influenza A. The susceptibility of people to a new variety of influenza A virus is also affected by a recent illness caused by an old variety. (Slepushkin 1959). He reported that in the summer outbreak caused by A2 virus in Poland the sickness rate for those who had been ill in the spring with A1 was 4.7% whilst the rate for those who were not affected in the spring was 10.5%. However, this immunity was found to be short-lived when examined in the light of another outbreak during the fall.

5. POPULATION SURVEY OF INFLUENZA

Estimates of number of cases in an epidemic is based on reports of excess deaths from influenzal pneumonia, excess respiratory illness, data obtained from questionnaires and/or from the results of laboratory surveys (Jensel et al 1958). The correct determination of the incidence of influenza in a general population from questionnaires is very difficult, due to inaccuracy in answering the questions (Horsfall and Tamm 1965). Influenza also often escapes diagnosis, either because it is mistaken for the febrile phase of other diseases, like malaria or because the disease runs such a mild course that it is not treated in hospital (Depoux et al 1959), or a physician is not consulted.

(i) Laboratory Diagnosis

The laboratory examination of material involves virus isolation and serology. Virus isolation is done by inoculation of prepared specimen into tissue cultures or embryonated eggs (Robinson 1964). This is however expensive and time consuming as compared to serologic methods (Pereira et al 1967). The serologic diagnosis of influenza virus infection can

be accomplished by virus neutralization test, haemagglutination inhibition test and the complement fixation test. Horsfall and Tamm (1965) remarked that serologic diagnosis is based on the fact that recovery from influenza virus infection is accompanied by the development of antibodies to the virus. Since a large proportion of the population possess demonstrable antibodies as a result of earlier infection with related strains, two specimens of sera are usually obtained, one in the early acute phase and another in convalenscence. These sera are titrated for antibody levels and current infection is diagnosed by a four-fold rise in the convalescent serum as compared with the acute phase serum.

Virus neutralization test is done by inocculating into susceptible host e.g. tissue culture or chick embryo, mixtures of serially diluted serum and virus suspension which have been incubated at a specific temperature for a specific time (Kwapinski 1965). The serum titre is expressed as the highest dilution which neutralizes the virus activity (Cytopathic effect or death of the embryo) in the tissue culture or the chick embryo.

The haemagglutination inhibition test depends on the ability of serum to prevent agglutination of erythrocytes by the test virus. Serial dilutions of the serum are incubated with equal volumes of the virus after which the erythrocyte suspension is added to the mixture, and the pattern of agglutination observed. The highest serum dilution causing complete inhibition of haemagglutination is taken as end point, and the titre is expressed as the reciprocal of the final serum dilution (Kwapinski 1965).

Some complexes of homologous antibodies and virus fix complement. When sensitized red blood cells suspension is added to such a mixture no haemolysis occur; absence of the homologous antibody in the serum leaves the complement free which causes haemolysis of the sensitized cells. The highest dilution of the serum which fixes complement under the test conditions, hence preventing haemolysis, is taken as the end point and the titre is expressed as the reciprocal of that dilution (Horsfall and Tamm 1964).

ii) Serum Survey

Examination of serum samples from various segments of the population for antibody spectrum to influenza viruses is very important. Surveys of this nature

help in judging past occurrence of a particular strain of virus and in appraising the probable future occurrence of the virus in the population (Hilleman et al 1958).

a) <u>Predictive Survey</u>:

Predictions of the likelihood of influenza epidemics are based on what is known of the percentage of the population at risk and on the identity of the circulating virus strain (Pereira et al 1967). Zhdanov (1967) reported that three epidemic waves of influenza had been predicted in the USSR by assessing the antibody levels of the population using current strains of the virus. From data available as to when the last outbreak of that influenza type occurred and what was happening in other countries, predictions were made. Early in 1966 there was an increase in influenza incidence which was attributed to influenza B. The last outbreak of influenza in the USSR had been recorded in 1962. In late 1965 and early 1966, epidemics of influenza B were recorded in Europe and America. Serological examination in March 1966 showed that the population had low levels of immunity against the B viruses circulating and on this information an outbreak was predicted. Pereira et al (1967) reported

on a survey of influenza antibody in England using the current strains of influenza viruses. They concluded that if the presence of circulating antibody was accepted as indicating some degree of resistance to influenza then a substantial proportion of the population was protected against the current strains. An outbreak of influenza was however recognized in England and viruses isolated were shown to be strains of influenza A2, not very different from the strains used by Pereira et al in 1967. (Report - Brit. Med. Journal Vol.1, 1968.

(b) Retrospective Survey: Rhodes and Van Rooyen (1968) remarked that studies on random samples of serum show that the average influenza antibody level rises following an epidemic. Analysis of such increases will therefore enable one to determine what type of virus is involved in an outbreak. Davenport et al (1953) and Francis (1953) reported that after influenza epidemics increases in antibody titres are also noted to strains prevalent in earlier infection and that the distribution of antibody is orientated in the various age groups to the particular virus predominant during early life of those individuals.

Mulder and Masurel (1958) by serology indicated strongly that the Asian strain of influenza may have been a recurrence of the virus type responsible for the pandemic in 1889-90. They examined sera collected before the 1957 outbreak in Netherlands and found that those who were 70 years and over had the highest antibody titres against the Asian strain. Serologic evidence has also been presented that the swine virus was related to a wide-spread human pathogen in 1918 and may have represented an A-type influenza virus prevailing during the 1918 pandemic (Shope 1938; Davenport et al 1953). A study on the range of immune bodies in people of various age groups therefore helps to reconstruct the natural history of evolution of influenza viruses.

PART I - SEROLOGIC SURVEY OF INFLUENZA ANTIBODIES

MATERIALS AND METHODS

(i) Sera: Each week from 14th November 1967 to 25th May, 1969, 50 sera were randomly collected from the Biochemistry Laboratory of the Civic Hospital. In all 3,400 serum samples were collected and titrated by the complement fixation method. These samples were among those received by the Biochemistry Laboratory for various tests.

Sera were collected into glass tubes after centrifugation of the blood samples at 1800 rpm. for about 20 minutes. These were stored, rubber-stoppered at -20°C.

1:4 dilutions of sera were made in diluent (VBS) and inactivated at 56°C for 30 minutes before titrations. In some cases inactivated sera were stored at -20°C ready for use. Dilutions were made using 1ml. pipettes but later on automatic pipettes were used.

(ii) Diluent: Veronal Buffered saline (VBS) pH 7.2 was prepared according to the modified formula of the Virus Reference Laboratory, Colindale, London, England, (see appendix). Five times concentrated stock solution made, was dispensed into Roux bottles with

screw cap stoppers and stored at +4°C. Working solution was prepared from time to time by diluting the stock solution 1:5 with distilled deionized water.

(iii) Haemolysin: Commercially prepared anti-sheep haemolysin, preserved in glycerin was ordered from the Slyvana Co., New Jersey. Titration was done according to Kwapinski (1965) with some modifications. 1:100 dilution was prepared from the original solution. Calculated volumes of diluent were placed in 9 tubes and the required volumes of 1:1000 dilution of haemolysin subsequently prepared were added to the tubes to give dilutions ranging from 1:1000 to 1:20,000. 0.2 ml. of each dilution was mixed with equal volume of 2%, 3% and 4% sheep red blood cells suspensions respectively and incubated at 37°C for 10 minutes to produce sensitized cells.

0.2 ml. of the sensitized cells at each dilution and cell concentration was added to sets of tubes containing 0.4 ml. each of diluent (to replace the antigen and antiserum solutions employed in the final complement fixation test) 0.2 ml of 1:30 dilution of complement solution was added to each tube and incubated at 37°C for 30 minutes. Controls for red blood cells suspensions were set up.

The titre of the haemolysin was estimated in terms of 100% haemolysis and the last tube showing complete haemolysis was taken as the end point with that dilution of haemolysin as one minimal haemolytic dose (1 MHD). The titration gave the combination of haemolysin dilution and red blood cells concentration optimal for the experiment. 3 MHD of haemolysin and 2% red blood cells suspension were selected and used in the test proper. 3 MHD of haemolysin gave maximum sensitization of cells without causing agglutination.

(iv) Sheep red blood cells: Cells preserved in Alsever's solution were received from time to time from the Animal Diseases Research Institute in Hull and stored at +4°C. Portions of the cells were removed and washed with diluent everytime a test was done. The cells were washed with about thrice their volume by suspending in diluent and centrifuging. The supernatant fluid was discarded and washing repeated until the fluid was colourless, (in most cases after two cycles). Approximately 10% suspension was made by resuspending measured amounts of cells in required volume of diluent. The exact concentration of the 10% suspension was checked using Fisher electrophotometer and a standard graph. 2% suspension of cells

was then prepared for use in the test. Sensitized sheep cells suspension (haemolytic system) was prepared by incubating equal volumes of 2% sheep cells suspension and 3 MHD of haemolysin at 37°C for 10 minutes.

(v) Antigens: The antigens were all supplied freeze-dried in ampoules by the Laboratory of Hygiene, Ottawa, through the Virus Laboratory, Civic Hospital, except the Hong Kong strain antigen which was prepared by the Virus Laboratory.

(a) Virus strains: During the first part of the experiment the sera were tested against Influenza A soluble antigen (AS), Influenza A2/Canada/9/66 (AV), Influenza B soluble Lee strains (BS) and Influenza B/Canada/5/66. (BV). Later on, the B antigens were substituted with Influenza A2/Singapore/1/57 and Influenza A2/Hong Kong/68.

(b) Titration of Antigens: The antigens were reconstituted as directed. Progressive dilutions starting from 1:2 were made in tubes using the diluent. These were each titrated quantitatively for specific complement fixation by the chess-board method in plastic trays against antisera purchased from Microbiological Associates, Maryland. 0.25 ml

volumes were used. Initial incubation for complement fixation was done at +4°C overnight (18hrs.) and then at 37°C for 30 minutes for the haemolytic system. The dilution of antigen which fixed not less than 75% complement (1:30) with the highest dilution of its antiserum was taken as the working dilution and this represented one optimal dilution (1 MHD) used in the test proper. Antigen dilutions remaining after each experiment were kept frozen at -20°C for later use.

(vi) Complement: This was purchased from Qualicom Laboratories and polled for titration. Dilutions from 1:50 to 1:300 were made in tubes by adding 1:10 solution of complement to the calculated volumes of diluent, (see appendix). The rest of the pooled sample was broken down into 0.5 ml. aliquots and stored frozen at -20°C.

0.2 ml. of each dilution was added to a mixture of 1 volume of diluent (representing anti-serum in the test) and 1 volume of the optimal dilution of antigen. Each antigen was used in the titration. Another set of tubes containing a double volume of the diluent as control was put up. The tubes were incubated at +4°C overnight (18 hrs.) after which they were allowed to

stand at room temperature for 10 minutes and then 0.2 ml. of 2% sensitized sheep cells added. These were reincubated at 37°C for 30 minutes. The end point was determined by finding the highest dilution of complement causing 100% haemolysis and this represented 1 MHD of complement. Further titration was done to determine what level of MHD of complement would give the best reading. From the results, 3 MHD of complement was chosen and used throughout the experiment. A fresh dilution was made every day a test was done.

II. COMPLEMENT FIXATION TEST:

The complement fixation microtechnique described by Sever (1962) was followed with some modifications. Linbro platinum Takatsy loops (0.025 ml. delivery) were purchased and calibrated according to the methods suggested by the manufacturers. The loops were dipped into weighed amount of saline and the difference in weight after withdrawal noted. The amount of saline taken by a loop should fall within specified range in order to have correct delivery. The loops were checked before each test by pre-wetting them in saline and touching the tips on the loop delivery tester blotter. Polypropylene Luer taper pipettes fitted with stainless steel

dropping tips (0.025 ml/drop) were used in delivering the reagents. 0.025 ml. unit volumes of reagents were used in Linbro disposable plastic plates with radial bottom cups. Sera were diluted using the loops, in two-fold steps starting from 1 : 8. Loops were placed in the cups of the plastic plates which have previously received one drop of diluent, with the first cups containing one drop of diluted sera as well. The styluses of the loops were rotated with the fingertips to ensure good mixing. The loops were then removed and rotated in successive cups until the last dilutions were done. Loops were then washed in two changes of 300 ml. distilled water, pre-wetted in saline and then blotted to discharge fluid before use in diluting another set of sera. The plates were covered with styrene sheets after reagents have been added and then incubated at +4°C overnight for fixation of complement. The plates were allowed to stand at room temperature for 10 minutes. 0.025 ml of sensitized cells suspension was added to each cup of the plates and then sealed with adhesive acetate sheets. The plates were shaken well and placed at 37°C for 30 minutes; plates were reshaken every 10 minutes during incubation. They were placed at 4°C after incubation for at least 2 hours by which time the non-haemolysed

cells have settled down completely to form a butt.

Results were read by placing the plates on a white background with a strong light overhead. Titres were recorded as the reciprocal of the highest serum dilution producing not less than 75% fixation. of complement with the antigen.

Controls for positive sera, test sera, antigens, complement and red blood cells were set up with every test.

Anti-complementary effect of serum was eliminated by diluting the serum above the non-specific fixation level which was determined by titration of a serum in the absence of the antigen (Kwapinski 1965). Actually this was the usual serum control put up for all the sera tested. When the anti-complementary effect was still not removed, 1 (one) volume of guinea pig complement was added to 3 volumes of serum and the mixture held at 4°C overnight and then at 37°C for 30 minutes. Sufficient diluent was added to give 1:4 dilution of the test serum and this was inactivated at 56°C for 30 minutes (Schmidt and Lennette 1965). The serum was then retitrated.

Antigen controls were done by performing the

test with the optimal dilutions of the antigens in the absence of serum; complement controls consisted of dilutions of complement at 3, 2, 1 and ½ MHD and two volumes of diluent. There should be complement haemolysis at the dilutions of 3 and 2 MHD and either complete haemolysis or trace of cells at 1 MHD. Complete haemolysis at ½ MHD was indicative that excess complement was used whilst partial haemolysis at 3 and 2 MHD meant insufficient complement was used in the test (Schmidt and Lennette 1965).

Red blood cells (RBC) controls consisted of sensitized sheep cells suspension and three volumes of diluent and this should show no haemolysis.

Results: As a result of work undertaken at the Virus Diagnostic Unit, Ottawa Civic Hospital, an outbreak of Asian influenza in Ottawa was predicted for the Winter of 1967 and the following spring, based on the fact that it had been at least three years then, since Ottawa experienced a major outbreak of Asian 'flu which is known to occur in North America at intervals of two to three years; and also due to the fact that the virus was present in the community which had a low state of immunity during May 1967 (Phipps and Westwood, 1967, unpublished).

The present study was therefore undertaken as a survey to follow the incidence of influenza during the period of the expected outbreak and to determine the value of serological procedures both in predicting and following the course of an outbreak. Fig. 1 shows the overall picture as it emerged over the 17 month period of the study. The graph shows the percentage of sera positive to influenza soluble (AS) and viral (AV) antigens at $\geq$1:8 dilution and suggests that the 1967 outbreak occurred as predicted and extended from December 1967 to March, 1968.

The peak found during January 1969 was due to the outbreak of the Hong Kong 'flu epidemic which will be discussed later.

Preliminary examination of graphs using percentage positives to AS and AV separately and in combination at $\geqslant$1:8, 1:16, and $\geqslant$1:32 which are not shown here, indicated that there were no differences in these plots at the different levels, as far as the overall picture was concerned. Anti AS and AV combination was chosen and used thereafter. This combination has an advantage in that it eliminates certain possible errors. Positive anti AS only, would not show the influenza strain and likewise anti AV only, would not distinguish antibodies due to either past infection, immunization or recent infection.

Fig. 2 shows the pattern when the results are plotted weekly, bi-weekly and monthly. The weekly results were found to be too erratic suggesting that the sample size was too small. Although the monthly plot eliminates the erratic nature of the other two plots it was considered to have failed to show clearly the extent of the outbreaks. The bi-weekly plot was therefore chosen as being the most satisfactory compromise.

Of considerable interest is the hump which is evident at the different levels of $>$1:8, 1:16 and $>$1:32 during the summer of 1968. (Fig.3) The possibility of over sensitivity at 1:8 titre is perhaps ruled out since this hump was not eliminated at the two higher levels. This graph gives clear indication of a summer prevalence of influenza although clinically no outbreak was noticed. The phenomenom that influenza virus circulates in the summer unnoticed clinically is therefore illustrated in this study. Francis and Maassab (1965) and Andrewes (1967) reported that influenza viruses might be seeded into the population during the warm season ahead of an evident outbreak during the winter. Fig. 3 substantiates the summer incidence of influenza. There are some interesting points raised as a result of the summer prevalence of Influenza A2/Canada and the subsequent Winter incidence of the Hong Kong strain and these will be dealt with in the discussion.

During early autumn of 1968, WHO reports indicated that an outbreak of a new variant of influenza, the Hong Kong 'flu should be expected during the winter. Preparations were therefore

carried out to detect the outbreak as it occurred and the spike obtained during January 1969 (Fig.3) represents the height of that epidemic. Results of virus isolations of this new strain at the Civic Hospital done in collaboration with this project, are set out in table II. Most of the isolations were done within a short time; 8 out of the total of 16 isolates were obtained from specimens received between 24th to 30th December, 1968. Fig. 4 shows the picture when the graph of percentage positives are superimposed on the histogram of the isolations. The peak level in the line graph was obtained seven days after the peak in the histogram. CF antibodies are usually detectable 5 - 7 days after influenza infection and this correlation between antibodies and infection obtained in this study fits the expected pattern.

A large proportion of each age group was found to be positive at $\geq$1:8 titre to the current A2 strain during the pre-epidemic seasons of 1968 and 1969, as shown in Tables III and V and Figs. 5 and 7. The relative increases in anti AS positives by age groups during the two epidemic periods are illustrated by Figs. 6 and 8. The greatest increases occurred in the age groups which had least pre-epidemic anti AV positives.

During the 1968 pre-epidemic season the age group 19 - 23 years showed the least anti AV positives and this group showed an increase of 26% which was the highest as compared to increases in age groups 24 - 40 and 41 - 65 years respectively during that same period (Fig. 6). The increase in age group 0 - 4 years can be explained by error due to sample size. Similar results were obtained during 1969 period (Fig. 8) and the relative high increase in the old age groups may be due to the outbreak of the new Hong Kong strain, since old people are usually more susceptible to new strains.

Jensen et al (1958) reported that presence of antibodies in individuals to influenza viruses without reasonable chance for contact with these viruses reflects antigenic crossing. In Fig. 9 10 and 11 and Tables VII, VIII and IX the sera used were obtained before the outbreak of the Hong Kong strain in Ottawa. The results therefore show that despite antigenic shift in influenza A strains since 1957, there is considerable cross-reaction between the strains of influenza A. The numbers of sera showing identical titres in each combination are enclosed in the square boxes. Addition of these

numbers gives the extent of cross reaction. It will be seen that A2/Singapore and A2/Canada show 54% identity (Table IX) whilst A2/Canada and A2/Hong Kong show 42% identity, and A2/Singapore and A2/Hong Kong show 46% identity.

In Table X and Fig. 12 presence of antibody to Inf. B is shown to be relatively infrequent in the sera obtained from pre-school age group with gradual increases thereafter. The peak level is obtained among young adults. Fig.13 shows the result of 100 pooled sera and this shows increases in influenza B antibody levels as influenza A average titre increases and this phenomenom is further illustrated in Fig. 14.

Table XI shows the correlation between anti-AS and anti-AV CF titres which indicated that anti-AV is usually higher than anti-AS. 72% of the sera were positive to both AS and AV at titres equal or greater than 1:8 which suggests that during recent infection with influenza, anti-AS is usually accompanied by anti-AV.

Relationship between climate and incidence of influenza is shown in Table XII. High levels of

positives to AS were obtained during the cold months indicating high incidence of infection. The effect of humidity is felt when in combination with high temperature and this point is further illustrated by Table XIII which shows that at high temperature, differences in relative humidity have marked effect on the survival of the influenza virus.

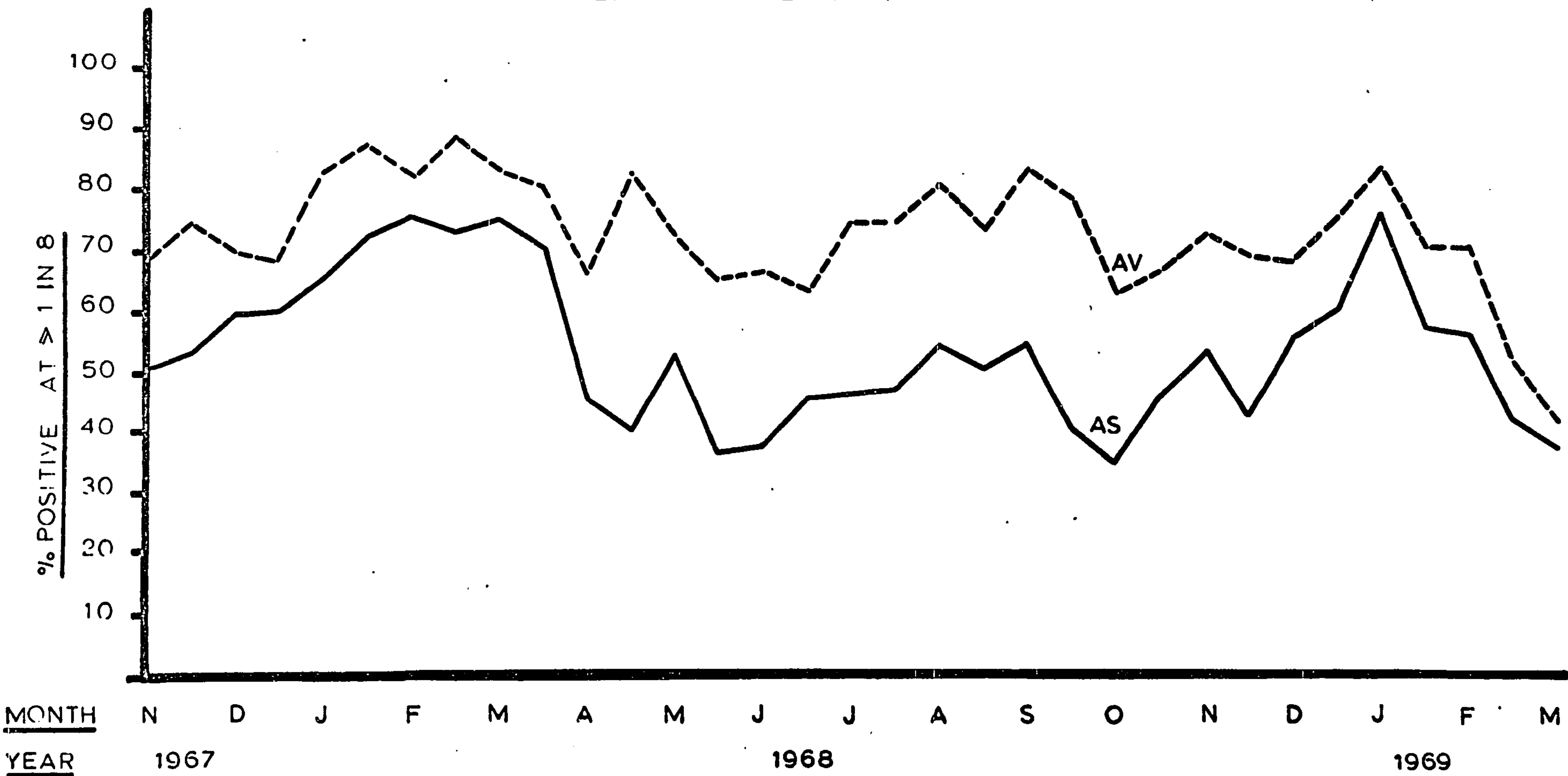
FIG. 1
INFLUENZA ANTIBODIES IN THE POPULATION OF OTTAWA.
BI-WEEKLY PLOT.
100
90
80
70
60
50
40
30
20
10
% POSITIVE AT ⩾ 1 IN 8
AV
AS
MONTH
N D J F M A M J J A S O N D J F M
YEAR
1967
1968
1969

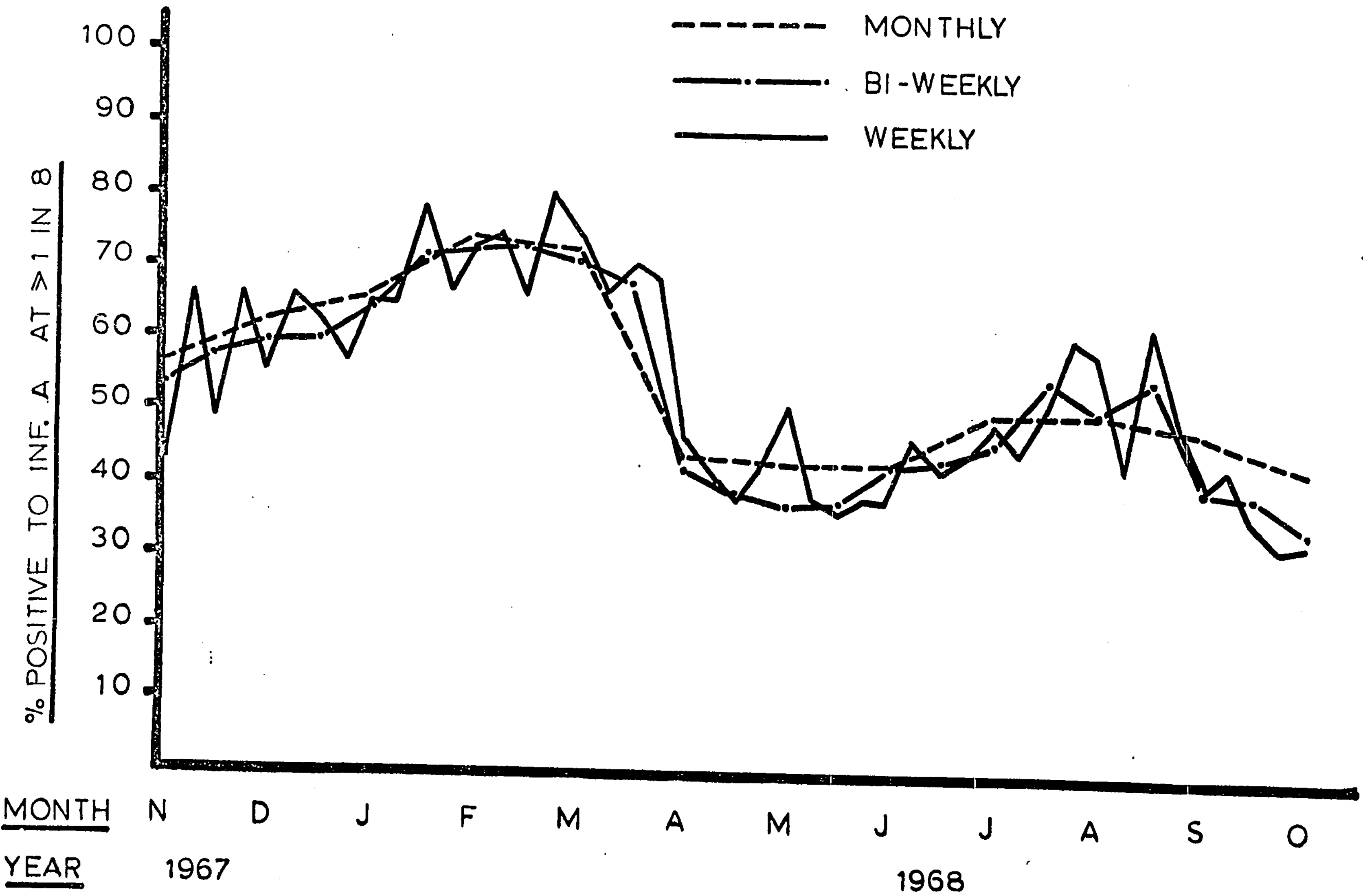

FIG. 2
INFLUENZA ANTIBODIES IN THE POPULATION OF OTTAWA.
MONTHLY
BI-WEEKLY
WEEKLY
100
90
80
70
60
50
40
30
20
10
% POSITIVE TO INF. A AT ≥ 1 IN 8
MONTH
N D J F M A M J J A S O
YEAR
1967
1968

FIG. 3 INFLUENZA A ANTIBODIES IN THE POPULATION OF OTTAWA.

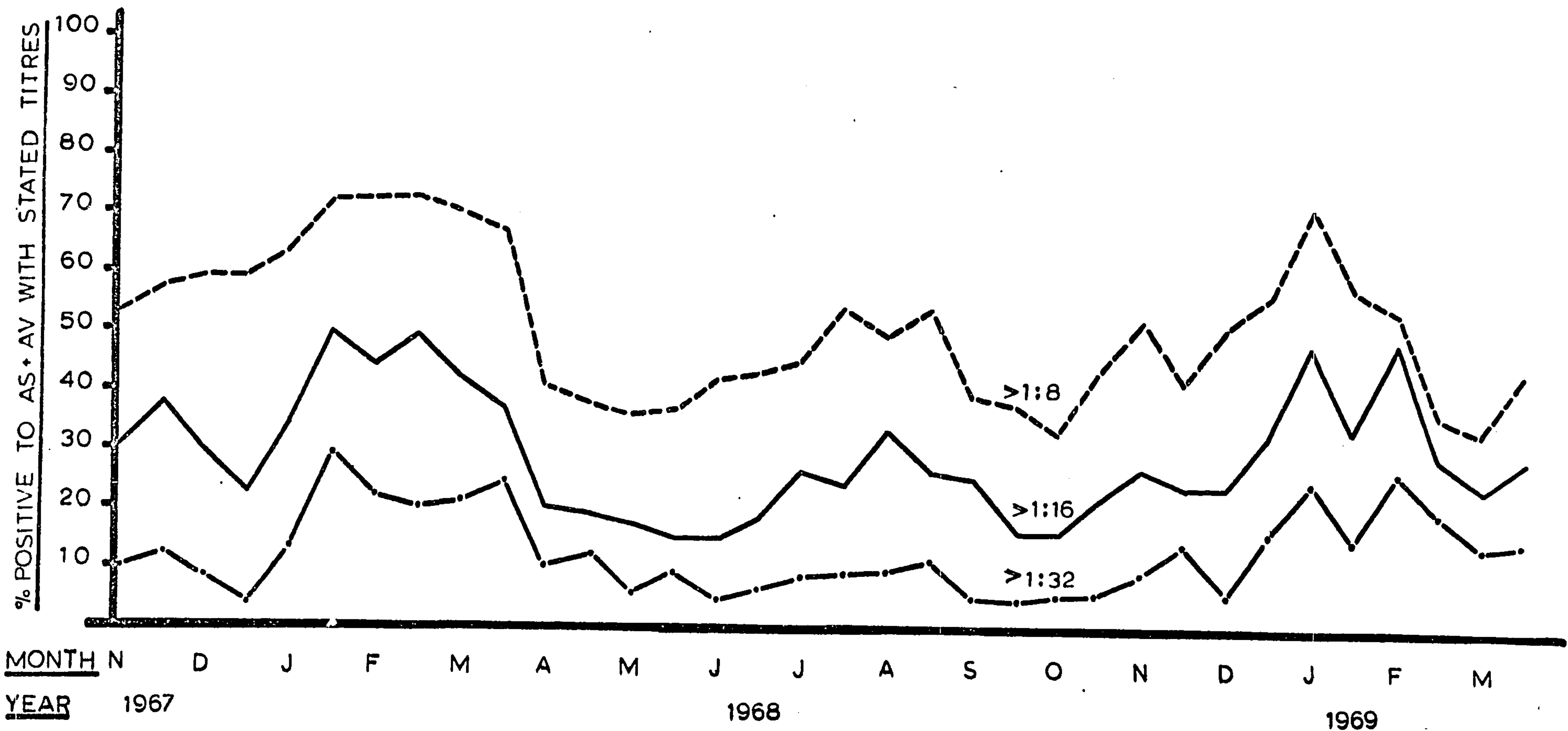

TABLE II

* INFLUENZA A2/HONG KONG ISOLATIONS AT THE VIRUS LABORATORY, CIVIC HOSPITAL

Isolation No.	Date Specimen was received
1	16th December, 1968
2	18th December, 1968
3	19th December, 1968
4	19th December, 1968
5	24th December, 1968
6	24th December, 1968
7	24th December, 1968
8	27th December, 1968
9	30th December, 1968
10	30th December, 1968
11	30th December, 1968
12	30th December, 1968
13	7th January, 1969
14	13th January, 1969
15	15th January, 1969
16	21st January, 1969

* By courtesy of the Laboratory Scientist, Virus Laboratory, Civic Hospital

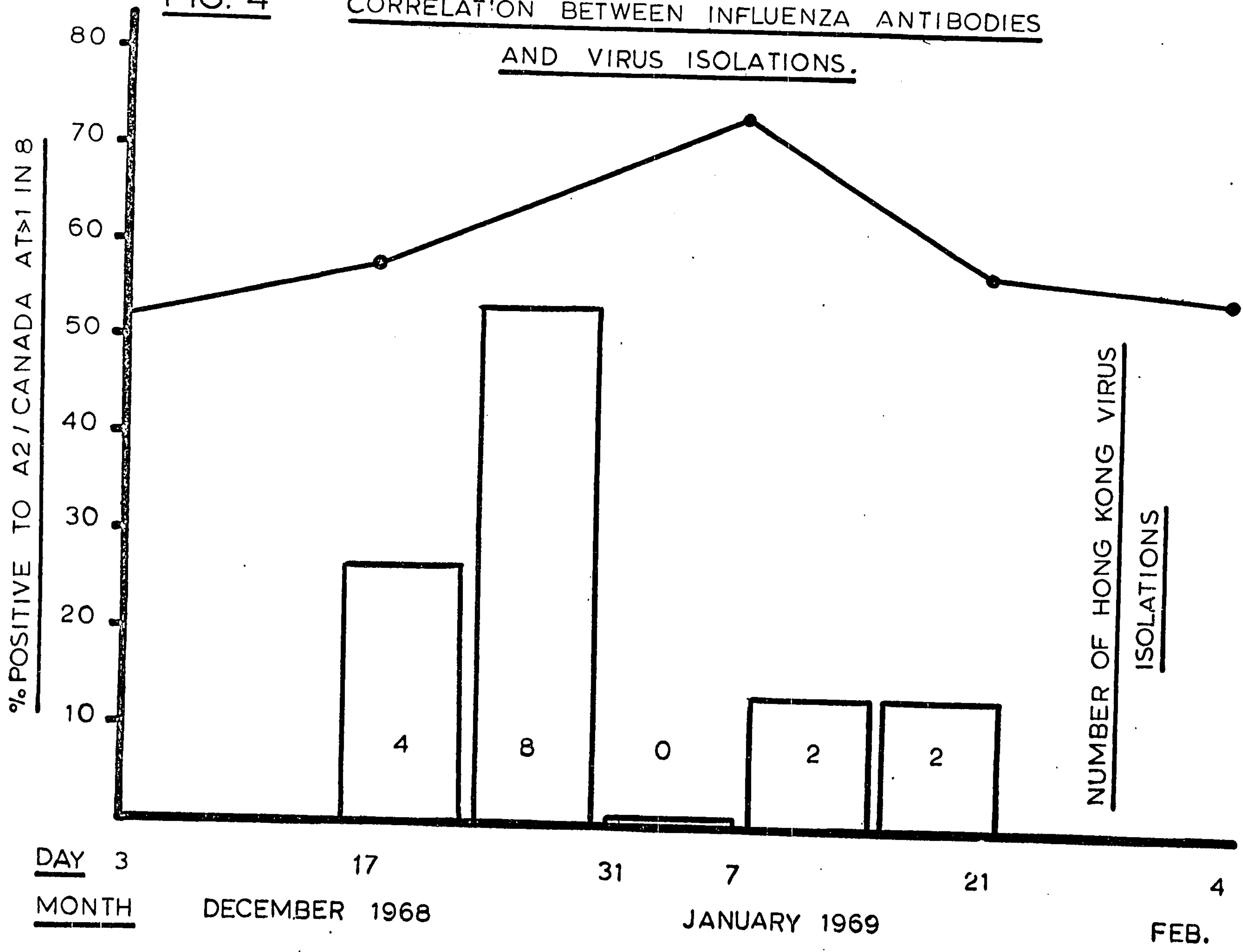
FIG. 4
CORRELATION BETWEEN INFLUENZA ANTIBODIES AND VIRUS ISOLATIONS.
% POSITIVE TO A2/CANADA AT≥1 IN 8
80
70
60
50
40
30
20
10
4
8
0
2
2
NUMBER OF HONG KONG VIRUS ISOLATIONS
DAY 3
17
31
7
21
4
MONTH
DECEMBER 1968
JANUARY 1969
FEB.

TABLE III

Specific Antibody to Influenza A soluble and
Influenza A2/Canada/9/66
14th November 1967 - 4th January, 1968

Age Group in years	ANTIBODY TITRE*												TOTAL
	ANTI AS						ANTI AV						
	< 8	≥ 8	8	16	32	≥64	< 8	≥8	8	16	32	≥64	
0-4	2 100%	0 0%	0	0	0	0	1 50%	1 50%	0	0	1	0	2
5-12	12 75%	4 25%	1	2	1	0	5 31%	11 69%	1	4	5	1	16
13-18	6 46%	7 54%	3	3	1	0	3 23%	10 77%	1	4	3	2	13
19-23	6 50%	6 50%	3	2	0	1	5 41.6%	7 58.4%	2	4	0	1	12
24-40	26 46.4%	30 53.6%	16	11	3	0	18 32%	38 68%	15	8	11	4	56
41-65	64 35.9%	114 64.1%	53	40	16	5	49 27.5%	129 72.5%	38	39	34	18	178
65+	41 43%	54 57%	21	21	10	2	33 34.7%	62 65.3%	17	22	18	5	95
NK	10 37%	17 64%	6	7	2	2	6 22%	21 78%	4	8	6	3	27

*Titre expressed as reciprocal of highest dilution of serum fixing not less than 75% complement.

Anti AS - antibody to soluble antigen
Anti AV - antibody to viral antigen
NK - not known

TABLE IV

Specific Antibody to Influenza A soluble
and Influenza A2/Canada/9/66
23rd January - 27th March, 1968

Age Group in years	ANTIBODY TITRE*												Total
	ANTI AS						ANTI AV						
	<8	≥8	8	16	32	≥64	<8	≥8	8	16	32	≥64	
0-4	3 42.8%	4 57.2%	2	1	1	0	3 42.8%	4 57.2%	1	0	0	3	7
5-12	13 61.9%	8 38.1%	5	1	1	1	8 38.1%	13 61.9%	2	2	2	7	21
13-18	10 62.5%	6 37.5%	4	1	1	0	8 50%	8 50%	1	4	1	2	16
19-23	4 23.5%	13 76.5%	7	2	2	2	0 0%	17 100%	2	4	7	4	17
24-40	23 30.6%	52 69.4%	27	11	8	6	15 20%	60 80%	8	20	17	15	75
41-65	41 19.9%	165 80.1%	64	47	34	20	24 11%	182 89%	31	60	49	42	206
65+	37 28%	98 72%	21	29	21	27	23 17%	112 83%	19	20	26	47	135
N.K.	5 21.7%	18 68.3%	6	9	2	1	4 17%	19 83%	3	5	8	3	23

* Titre expressed as reciprocal of highest dilution of serum fixing not less than 75% complement.
Anti AS - antibody to soluble antigen
Anti AV - antibody to viral antigen
N.K. - not known

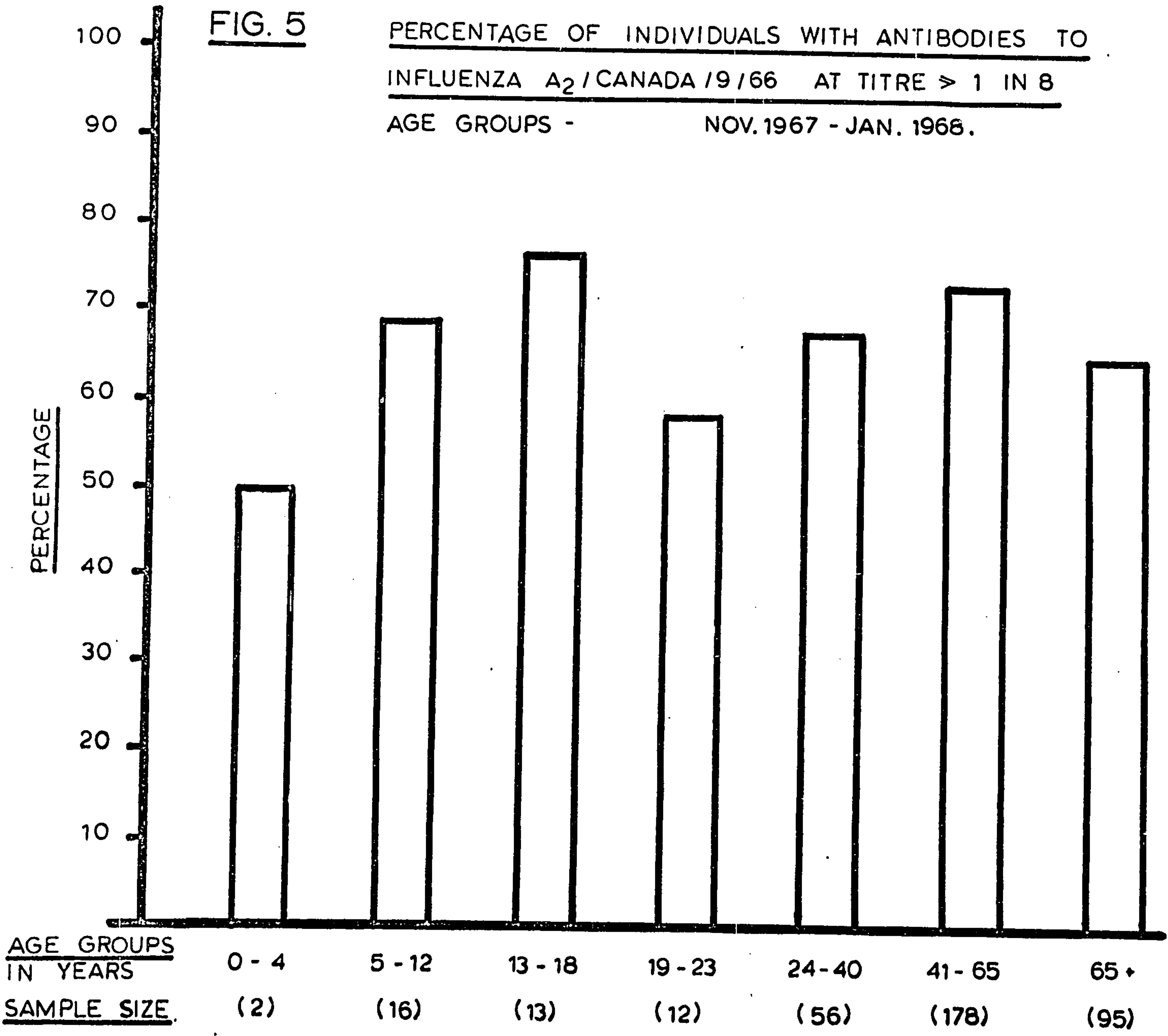
FIG. 5
PERCENTAGE OF INDIVIDUALS WITH ANTIBODIES TO
INFLUENZA A2 / CANADA / 9 / 66 AT TITRE ≥ 1 IN 8
AGE GROUPS - NOV. 1967 - JAN. 1968.
PERCENTAGE
100
90
80
70
60
50
40
30
20
10
AGE GROUPS IN YEARS
0 - 4
5 - 12
13 - 18
19 - 23
24 - 40
41 - 65
65 +
SAMPLE SIZE
(2)
(16)
(13)
(12)
(56)
(178)
(95)

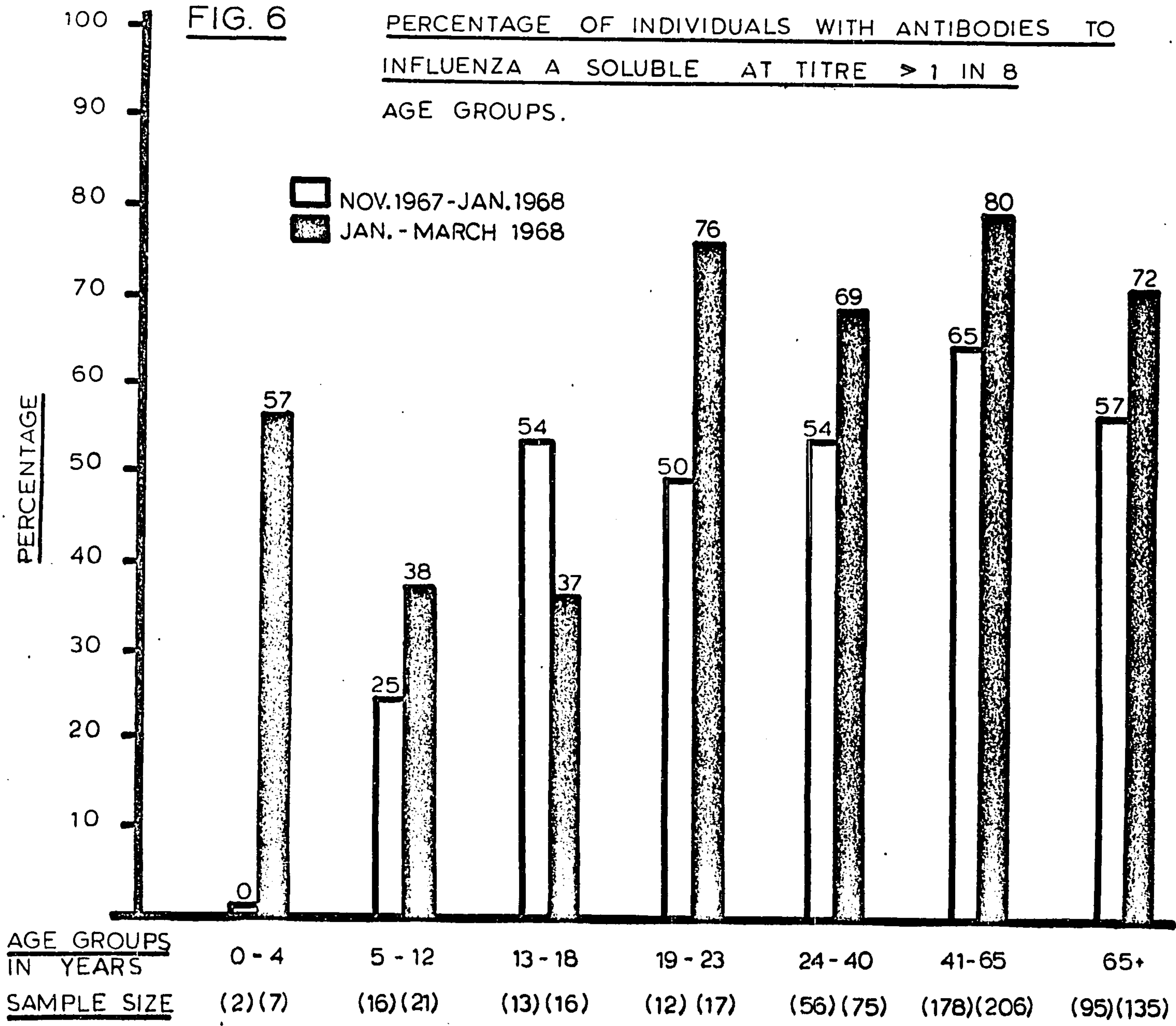
FIG. 6
PERCENTAGE OF INDIVIDUALS WITH ANTIBODIES TO INFLUENZA A SOLUBLE AT TITRE > 1 IN 8
AGE GROUPS.
NOV. 1967 - JAN. 1968
JAN. - MARCH 1968
PERCENTAGE
100
90
80
70
60
50
40
30
20
10
0
57
25
38
54
37
50
76
54
69
65
80
57
72
AGE GROUPS IN YEARS
0 - 4
5 - 12
13 - 18
19 - 23
24 - 40
41 - 65
65+
SAMPLE SIZE
(2) (7)
(16) (21)
(13) (16)
(12) (17)
(56) (75)
(178) (206)
(95) (135)

TABLE V

Specific Antibody to Influenza A soluble and Influenza A2/Canada/9/66
17th September - 5th November, 1968

Age Group in Years	ANTIBODY TITRE*												Total
	ANTI AS						ANTI AV						
	<8	≥8	8	16	32	≥64	<8	≥8	8	16	32	≥64	
0-4	6 85.7%	1 14.3%	1	0	0	0	3 42.8%	4 57.2%	0	2	0	2	7
5-12	13 92.8%	1 7.2%	0	0	0	1	9 64%	5 36%	1	1	0	3	14
13-18	13 72%	5 28%	2	1	1	1	5 27.7%	13 72.3%	2	3	3	5	18
19-23	13 68%	6 32%	4	0	2	0	8 42%	11 58%	3	2	4	2	19
24-40	37 53.6%	32 46.4%	22	8	2	0	18 26%	51 74%	10	19	16	6	69
41-65	77 54.6%	64 45.4%	29	24	9	2	44 31%	97 69%	29	29	31	8	141
65+	57 51.8%	53 48.2%	23	21	8	1	37 33.6%	73 66.4%	20	23	18	12	110
N.K.	16 72.7%	6 27.3%	3	2	1	0	10 45%	12 55%	4	5	1	2	22

* Titre expressed as reciprocal of highest dilution of serum fixing not less than 75% complement.
Anti AS - antibody to soluble antigen.
Anti AV - antibody to viral antigen
N.K. - not known.

TABLE VI

Specific Antibody to Influenza A Soluble and Influenza A2/Canada/9/66
November 1968 -- January 1969

Age Group in years	ANTIBODY TITRE*												Total
	ANTI AS						ANTI AV						
	<8	≥8	8	16	32	≥64	<8	≥8	8	16	32	≥64	
0-4	3 75%	1 25.0%	0	0	0	1	3 75.0%	1 25.0%	0	0	0	1	4
5-12	8 72.7%	3 27.3%	2	1	0	0	4 36.0%	7 64.0%	1	1	2	3	11
13-18	6 75.0%	2 25.0%	0	1	0	1	3 37.5%	5 62.5%	0	2	2	1	8
19-23	5 55.5%	4 44.5%	3	1	0	0	2 22%	7 78.0%	2	3	2	0	9
24-40	33 52.0%	30 48.0%	16	9	3	2	20 31.7%	43 68.3%	15	14	8	6	63
41-65	43 28.9%	105 71.1%	41	25	30	9	26 17.5%	122 82.5%	16	47	36	23	148
65+	26 28.5%	65 71.5%	23	24	11	7	22 24.0%	69 76.0%	19	23	15	12	91
N.K.	10 62.5%	6 37.5%	2	1	1	2	7 43.7%	9 56.3%	2	3	2	2	16

*Titre expressed as the reciprocal of the highest dilution serum fixing not less than 75% complement.
Anti AS - antibody to soluble antigen
Anti AV - antibody to viral antigen
N.K. - not known

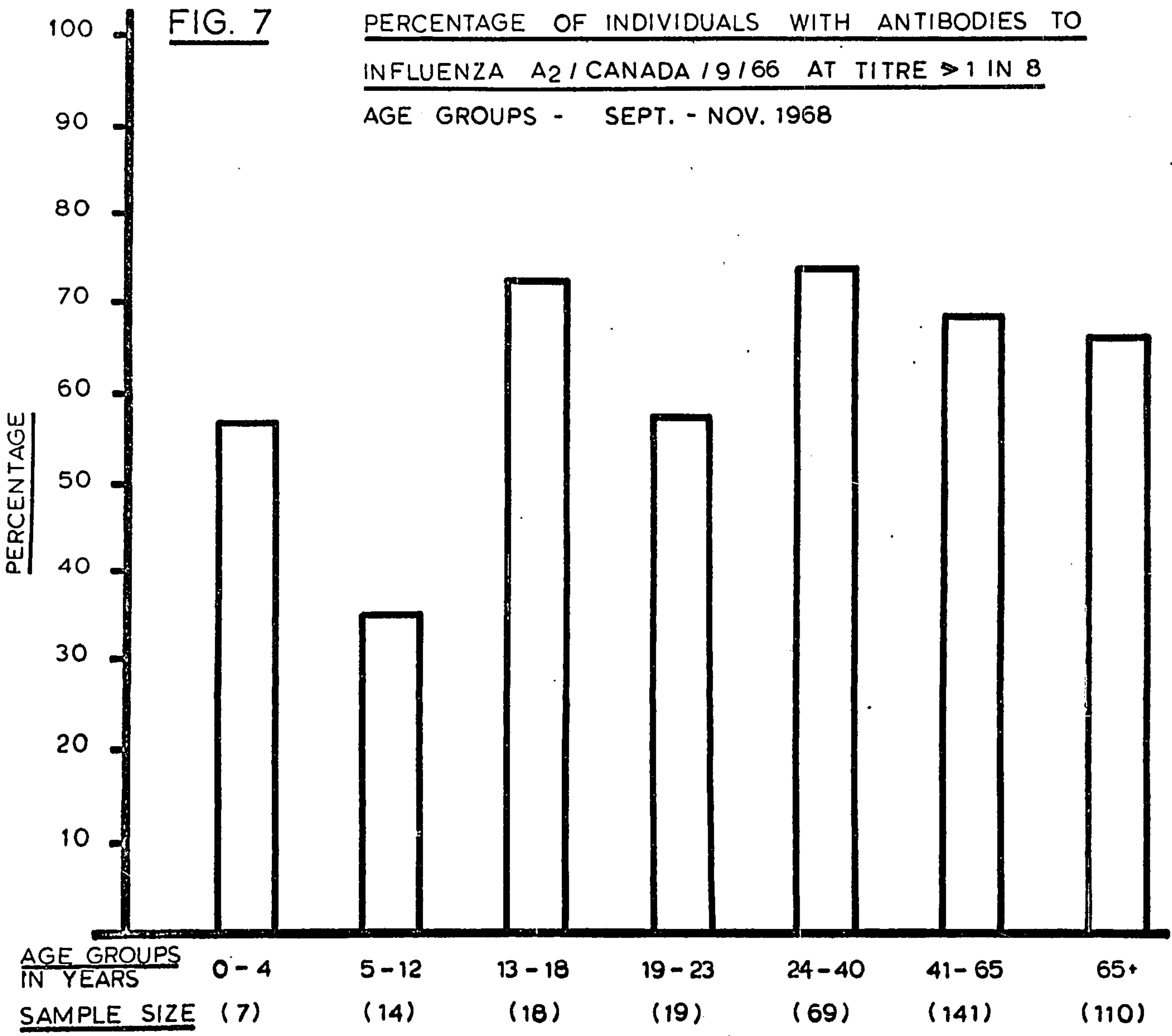
FIG. 7
PERCENTAGE OF INDIVIDUALS WITH ANTIBODIES TO
INFLUENZA A2 / CANADA / 9 / 66 AT TITRE ≥ 1 IN 8
AGE GROUPS - SEPT. - NOV. 1968
PERCENTAGE
100
90
80
70
60
50
40
30
20
10
AGE GROUPS IN YEARS
0 - 4
5 - 12
13 - 18
19 - 23
24 - 40
41 - 65
65+
SAMPLE SIZE
(7)
(14)
(18)
(19)
(69)
(141)
(110)

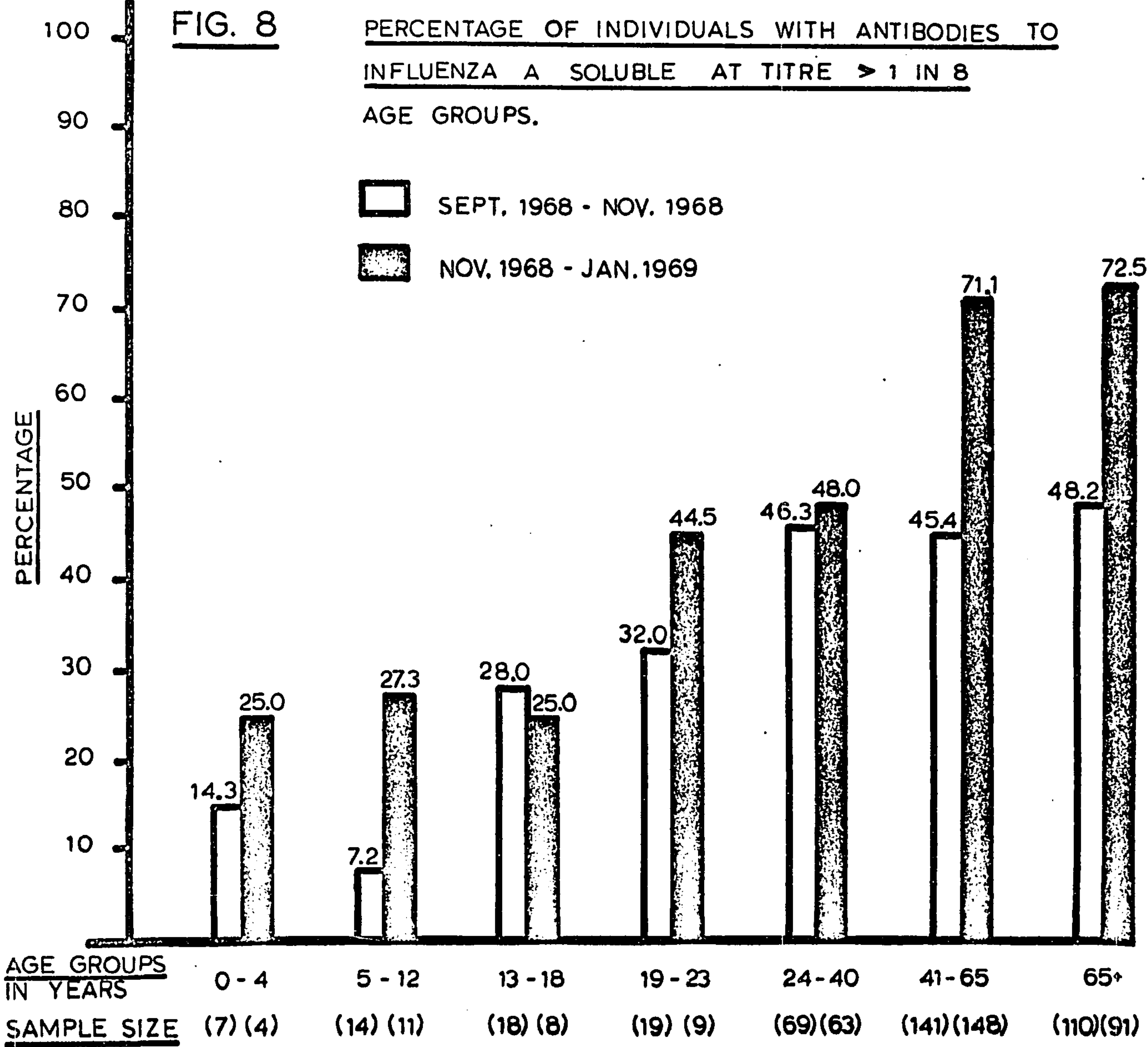
FIG. 8
PERCENTAGE OF INDIVIDUALS WITH ANTIBODIES TO
INFLUENZA A SOLUBLE AT TITRE > 1 IN 8
AGE GROUPS.
SEPT. 1968 - NOV. 1968
NOV. 1968 - JAN. 1969
PERCENTAGE
100
90
80
70
60
50
40
30
20
10
14.3
25.0
7.2
27.3
28.0
25.0
32.0
44.5
46.3
48.0
45.4
71.1
48.2
72.5
AGE GROUPS IN YEARS
0 - 4
5 - 12
13 - 18
19 - 23
24 - 40
41 - 65
65+
SAMPLE SIZE
(7) (4)
(14) (11)
(18) (8)
(19) (9)
(69) (63)
(141) (148)
(110) (91)

TABLE VII

Correlation between CF titres * of Influenza A2/Singapore/1/57 and Inf. A2/Hong Kong using sera collected 16th October, 1968

A2/Hong Kong/1/68	A2/Singapore/1/57 titre					
	<8	8	16	32	≥64	Total
<8	[12]	3	1	1	1	18
8	8	[5]	1	0	0	14
16	2	7	[2]	0	0	11
32	0	2	0	[3]	0	5
≥64	0	0	1	0	[1]	2
Total	22	17	5	4	2	50

* Titre expressed as the reciprocal of the highest dilution of serum fixing not less than 75% complement.

FIG. 9 ANTIBODY TITRES TO INF. A_2 / HONG KONG/1/68 AND A_2 / SINGAPORE /1/57

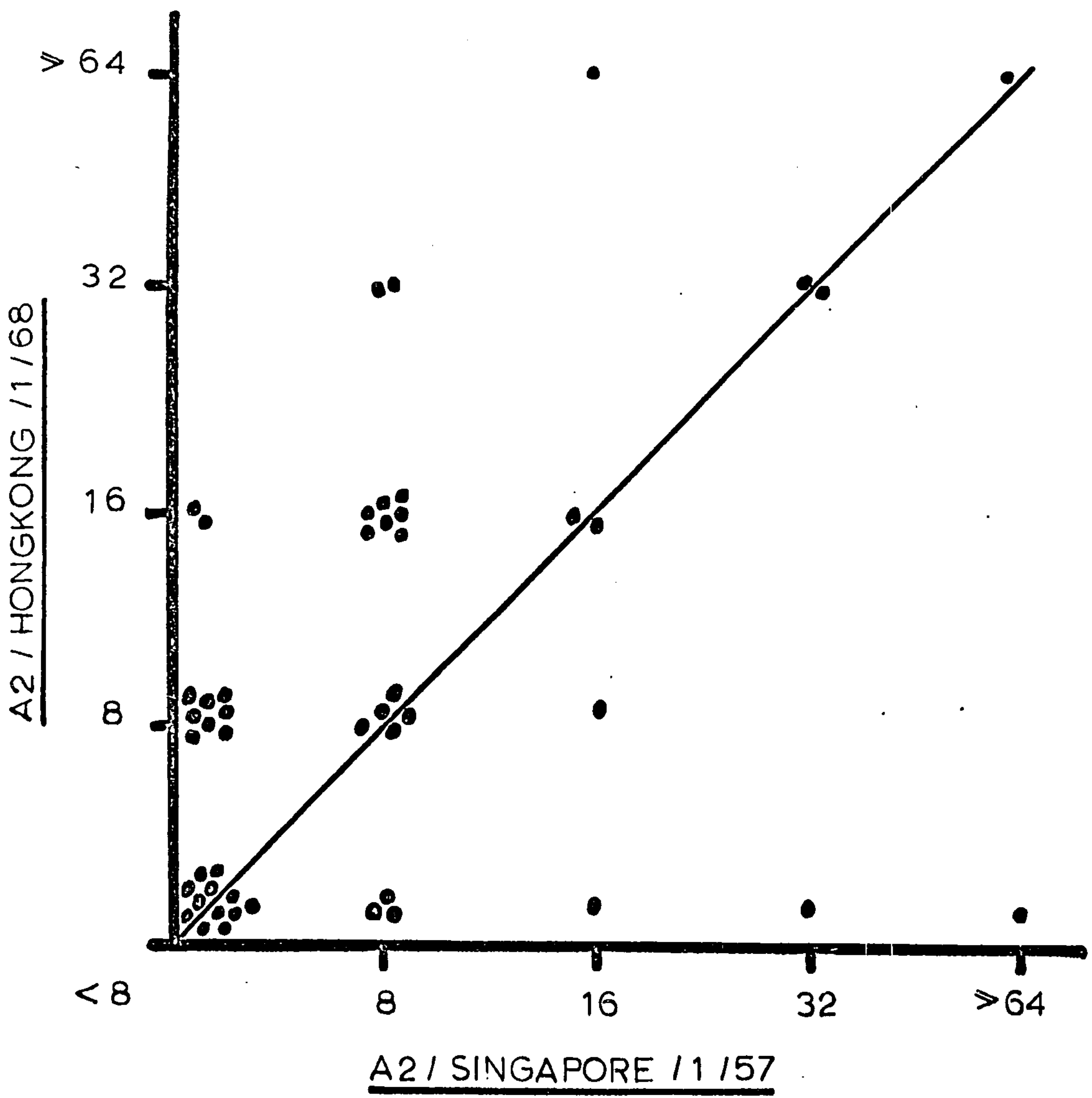

TABLE VIII

Correlation between CF Titres *
of Influenza A2/Canada/9/66 and
Influenza A2/Hong Kong using sera
collected 16th October, 1968

A2/Canada/9/66 Titre	A2/Hong Kong/1/68 Titre					Total
	<8	8	16	32	≥64	
<8	12	11	1	0	0	24
8	2	3	5	0	0	10
16	0	0	3	2	0	5
32	3	0	2	2	1	8
≥64	1	0	0	1	1	3
Total	18	14	11	5	2	50

* Titre expressed as the reciprocal of the highest dilution of serum fixing not less than 75% complement.

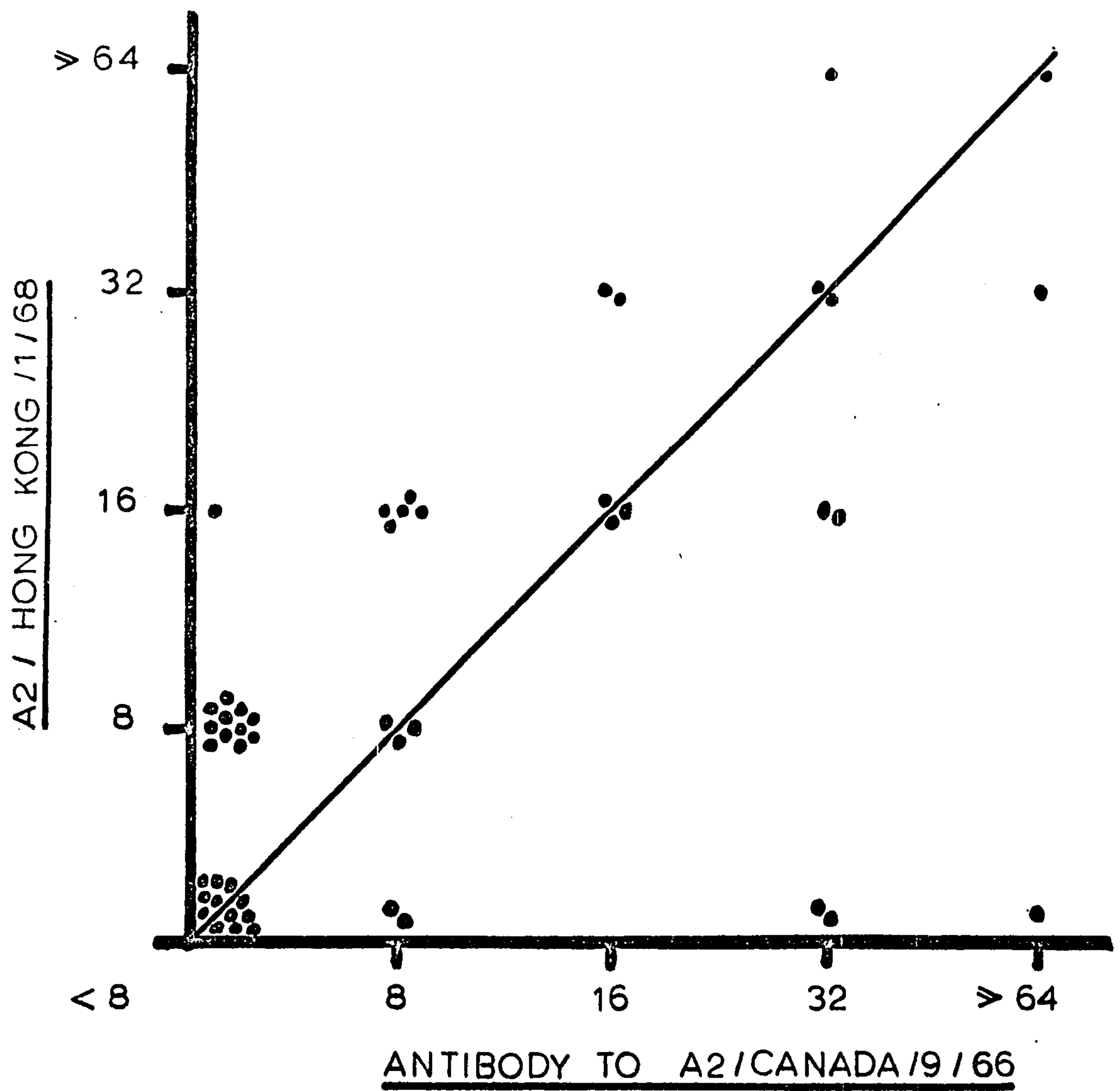

FIG. 10 ANTIBODY TITRES TO INF. A2 / HONGKONG /1/68 AND A2 / CANADA /9/66

TABLE IX

Correlation between CF Titres * of Influenza A2/Singapore/1/57 and Influenza A2/Canada/9/66 using Sera collected 16th October, 1968

A2/Canada/9/66 Titre	A2/Singapore/1/57 Titre					Total
	<8	8	16	32	≥64	
<8	17	7	0	0	0	24
8	4	4	2	0	0	10
16	0	4	1	0	0	5
32	1	2	2	3	0	8
≥64	0	0	0	1	2	3
Total	22	17	5	4	2	50

* Titre expressed as the reciprocal of the highest dilution of serum fixing not less than 75% complement.

FIG. 11 ANTIBODY TITRES TO INF. A2/SINGAPORE/1/57 AND INF. A2/CANADA/9/66.

A2/SINGAPORE/1/57

≥64
32
16
8
<8

8
16
32
≥64

A2/CANADA/9/66

TABLE X

Specific antibody to Influenza B/Canada/5/66
January - March, 1969

Age Group in years	ANTIBODY TITRE*												Total
	ANTI BS						ANTI BV						
	< 8	≥ 8	8	16	32	≥ 64	< 8	≥ 8	8	16	32	≥ 64	
0-4	7	0	0	0	0	0	7 100%	0 0%	0	0	0	0	7
5-12	20	1	0	1	0	0	14 66.6%	7 33.4%	4	2	1	0	21
13-18	15	1	0	1	0	0	13 81.3%	3 18.7%	1	1	1	0	16
19-23	11	6	1	4	1	0	6 35.3%	11 64.7%	2	5	4	0	17
24-40	47	28	17	4	5	2	44 58.6%	31 41.4%	11	12	5	3	75
41-65	119	87	42	29	13	3	90 43.2%	116 56.8%	43	36	31	6	206
65+	85	50	28	13	7	2	73 54.1%	62 45.9%	24	26	8	4	135
N.K.	18	5	3	2	0	0	14 60.8%	9 39.2%	3	6	0	0	23

* Titre expressed as the reciprocal of the highest dilution of serum fixing not less than 75% complement.

N.K. - not known

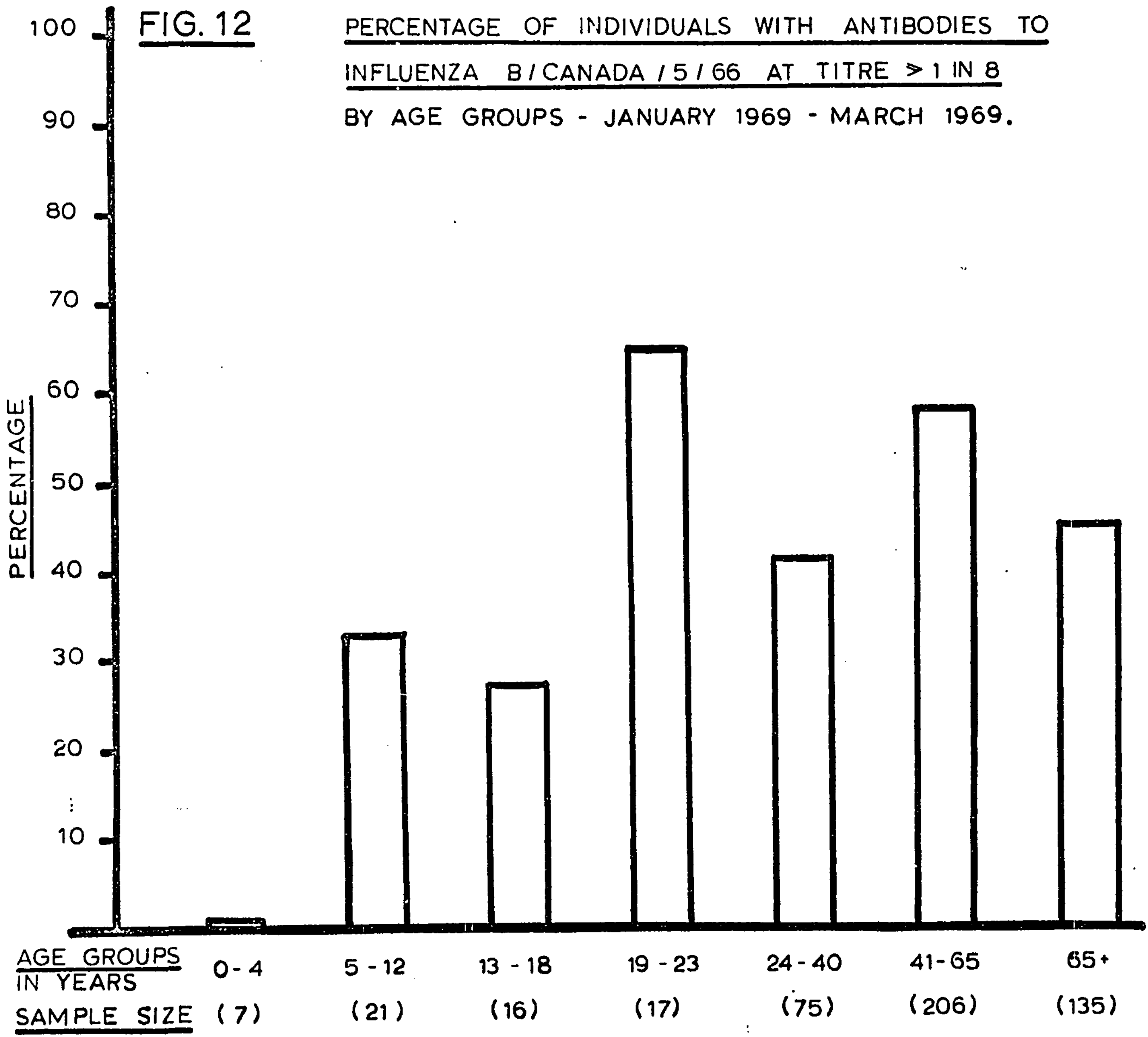
FIG. 12
PERCENTAGE OF INDIVIDUALS WITH ANTIBODIES TO INFLUENZA B/CANADA/5/66 AT TITRE ≥ 1 IN 8
BY AGE GROUPS - JANUARY 1969 - MARCH 1969.
PERCENTAGE
100
90
80
70
60
50
40
30
20
10
AGE GROUPS IN YEARS
0-4
5-12
13-18
19-23
24-40
41-65
65+
SAMPLE SIZE
(7)
(21)
(16)
(17)
(75)
(206)
(135)

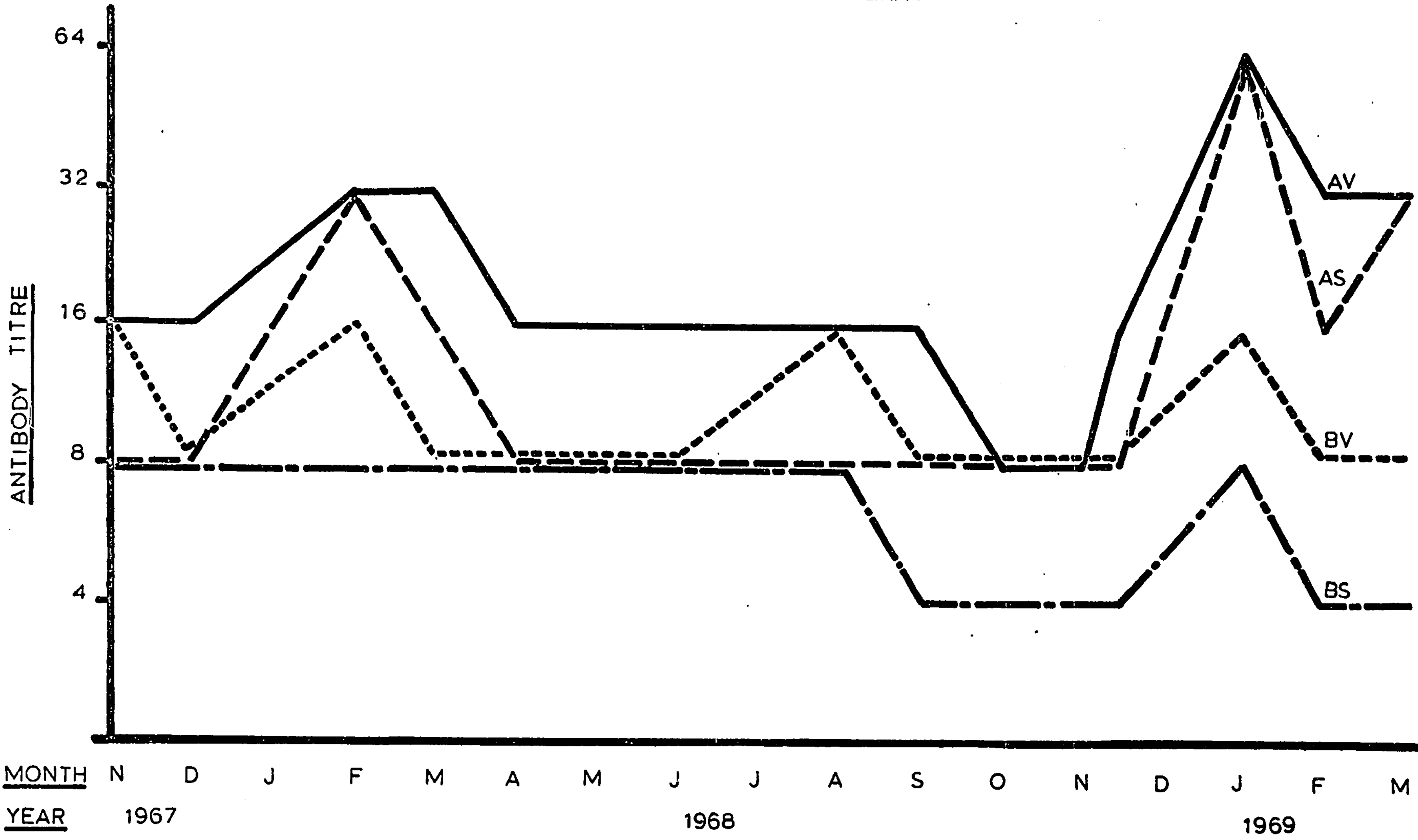

FIG. 13 INFLUENZA ANTIBODY LEVELS IN THE POPULATION OF OTTAWA.

RESULTS OF POOLED 100 SERA.

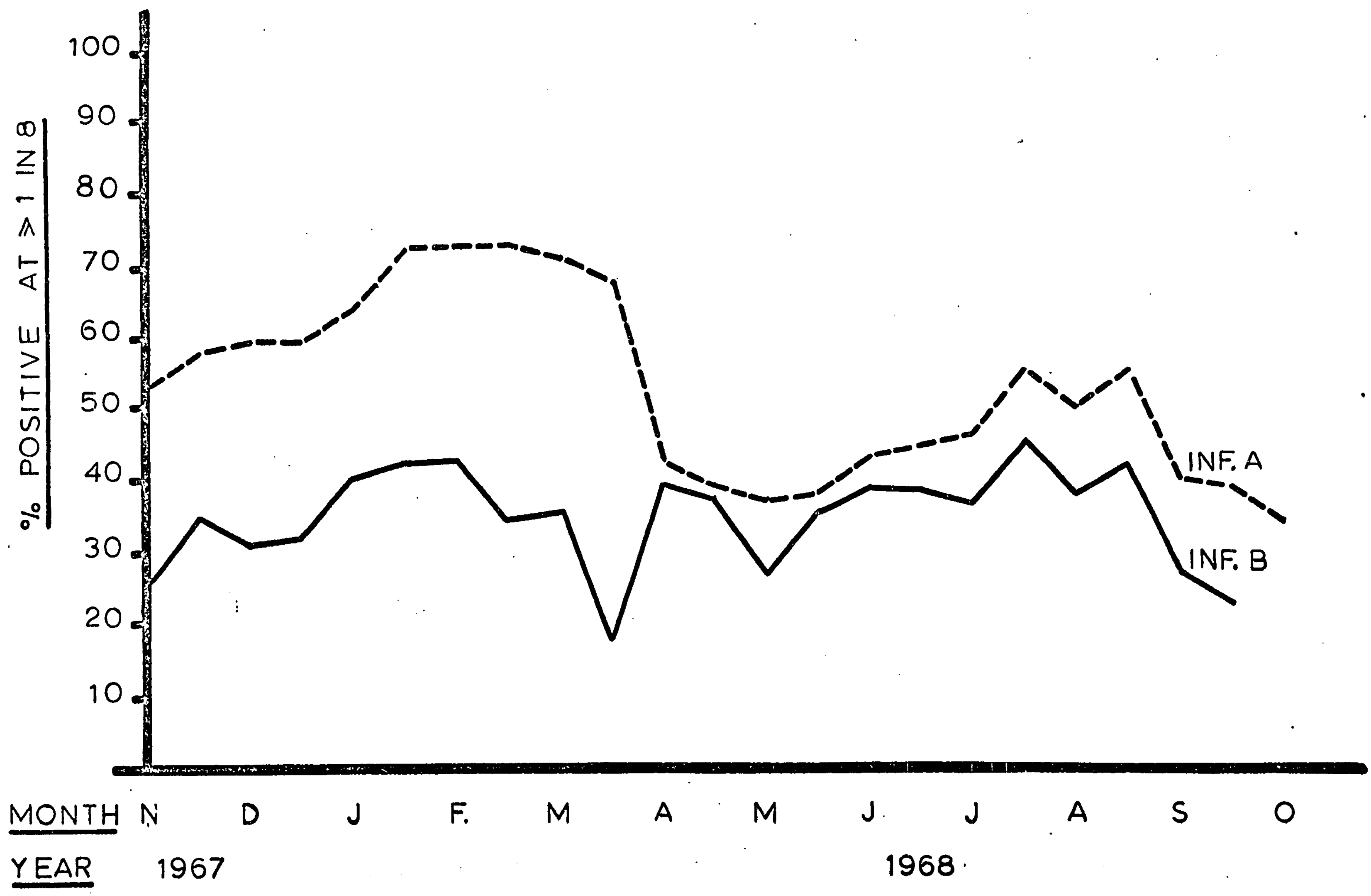

FIG: 14 INFLUENZA ANTIBODIES IN THE POPULATION OF OTTAWA.

BI-WEEKLY PLOT.

TABLE XI

Correlation between Anti"AS" and Anti"AV" CF Titres *

Anti AV \ Anti "AS"	<8	8	16	32	≥64	Total
<8	15	3	0	0	0	18
8	3	7	0	0	0	10
16	2	12	5	2	3	24
32	3	4	7	10	0	24
≥64	2	0	6	8	8	24
Total	25	26	18	20	11	100

* Titre expressed as the reciprocal of the highest dilution of serum fixing not less than 75% complement.

N.B.: 72% of the sera were positive to both AS and AV at titre≥ 8.

TABLE XI

Correlation between Anti"AS" and Anti"AV" CF Titres *

Anti AV \ Anti "AS"	<8	8	16	32	≥64	Total
<8	15	3	0	0	0	18
8	3	7	0	0	0	10
16	2	12	5	2	3	24
32	3	4	7	10	0	24
≥64	2	0	6	8	8	24
Total	25	26	18	20	11	100

* Titre expressed as the reciprocal of the highest dilution of serum fixing not less than 75% complement.

N.B.: 72% of the sera were positive to both AS and AV at titre ≥ 8.

TABLE XII

Relationship between *Climate and Incidence of Influenza

YEAR	MONTH	MEAN TEMPER-ATURE OF	RELATIVE HUMIDITY %	(a) % POSITIVE TO AS≥1/8
1967	Nov.	30.1	77.7	56
	Dec.	21.9	75.3	62
	Jan.	6.5	67.0	66
	Feb.	11.0	67.0	74
1968	Mar.	29.4	66.3	73
	Apr.	47.3	55.6	44
	May	53.5	59.6	43
	Jun.	61.5	71.6	43
	Jul.	68.9	69.6	50
	Aug.	64.0	65.6	50
	Sep.	61.9	75.3	48
	Oct.	49.5	76	44
	Nov.	30.5	79.3	45
	Dec.	15.5	77.3	67
1969	Jan.	15.5	76.3	63
	Feb.	20.0	73.0	41
	Mar.	25.0	67.6	51

* Information supplied by Dept. of Transport.

a - percentage positive at titre ≥8 to Influenza A soluble antigen.

TABLE XIII

Viability of Influenza A-PR8
after spraying *

Temperature °F	Relative Humidity %	% viable in hrs. ½	4	23
46	51	75	39	19
	82	71	39	3
68	51	49	6.4	Trace
	81	22	6.4	Nil
89	50	45	Trace	Nil
	81	15	Trace	Nil

* Harper G.J. (1961) J. Hyg. Camb. 59; 479

<u>Discussion</u>: Predictions of the likelihood of an epidemic of influenza have been based in the past, on antibody levels in the general population (Pereira et al 1967 ; Zhdanov 1967). From the present study it appears that a new approach can be made by analysing results obtained into age groups thereby assessing the relative risks of each group, hence the judicious application of vaccines. Such forecast should however take into consideration the possible emergence of new strains and their relationship to old and current strains of influenza. Slepushkin (1959) made an observation that the degree of susceptibility of people to a new variety of influenza virus is affected by illness caused by an old variety or related type. Resistance must therefore be allowed for making an epidemiological assessment of influenza sickness rate.

It could be seen that during the Hong Kong strain outbreak though pre-epidemic sampling showed that the older age groups were equally protected, there was considerable increase in anti-AS level in this group which means infection was high; presence of anti AS is indicative of recent infection (Fazekas de St. Groth and Donnelley 1950a).

Results of the degree of cross-reaction obtained in this work showed that people with A2/Singapore antibodies may be relatively better protected in an outbreak with A2/Hong Kong. 46% cross reaction was noticed between A2/Singapore and A2/Hong Kong as against 42% between A2/Canada and A2/Hong Kong. These differences in cross-reactivity can be taken as significant in view of the fact that A2/Canada was the current strain circulating at the time that the sera were taken and as such could raise its level of cross-reactivity. Other workers have reported similar results. Masurel (1969) examined the relationship between the Hong Kong virus and former human A2 isolates in human sera collected before 1957 in the Netherlands. He noticed that the same age groups showed high titres to both A2/Singapore/57 and the A2/Hong Kong/68 and suggested that these two strains might be related much more to each other than to the other strains used. Zalan and Labzoffsky (1969) also compared four strains of influenza A2 (A2/Singapore/57 (S/57); A2/Ontario/67 (O/67); A2/Hong Kong (WHO); A2/Ontario/68). They remarked that whereas O/67 and the two Hong Kong strains are related to S/57, O/67 is antigenically distinct from the two Hong Kong strains. These observations do not differ from ours and perhaps this explains

why there was such an increase in the old age group (Fig.8). Young adults had a better chance of being infected during their childhood with A2/Singapore/1/57 strain or related strains more so than with A2/Canada/9/66. Their antibodies might therefore be more related to A2/Singapore hence to A2/Hong Kong than A2/Canada and as such have better resistance to the new strain (Fukumi 1959; Davenport et al 1953).

More than 3,000 sera were tested during this work but the numbers of samples from most of the age groups were limited. A more selective investigation may therefore need to be done to assess the usefulness of predictions based on the suggested method.

The results of the pooled sera indicate that there were outbreaks of influenza A during the winter months of 1968 and 1969 in Ottawa since the average antibody levels to influenza A increased during that time (Rhodes and van Rooyen 1968).

Andrewes (1967) remarked that influenza virus might be seeded into the population during the summer ahead of a winter outbreak. In the present study a summer prevalence of A2/Canada/66 was noticed and this was followed by a winter outbreak. This outbreak was caused by the Hong Kong strain and not by A2/Canada. It was

interesting to observe that although A2/Canada virus was in the population during the summer, none was isolated when the Hong Kong strain outbreak occurred. It has been observed by many workers that when a new strain of influenza virus emerge the old strain disappears completely. From the results of this survey, it appears that this "strange" phenomenon happened once more.

The outbreak of the Hong Kong strain in the winter of 1969 made it difficult to see clearly the exact picture of what happens during the winter after a major outbreak of a strain had occurred in the previous winter and had been followed by a summer prevalence of the same strain. It can however be speculated that since no A2/Canada strain was isolated during the winter of 1969, a major outbreak caused by a strain the previous winter, produces an immune community such that although the virus might be present during the summer, the usual winter outbreak is not evident. This possibility might be the case since there is usually a time lapse of two to three years between major epidemics of influenza and also since immunity to a strain is known to last for about three years.

interesting to observe that although A2/Canada virus was in the population during the summer, none was isolated when the Hong Kong strain outbreak occurred. It has been observed by many workers that when a new strain of influenza virus emerge the old strain disappears completely. From the results of this survey, it appears that this "strange" phenomenon happened once more.

The outbreak of the Hong Kong strain in the winter of 1969 made it difficult to see clearly the exact picture of what happens during the winter after a major outbreak of a strain had occurred in the previous winter and had been followed by a summer prevalence of the same strain. It can however be speculated that since no A2/Canada strain was isolated during the winter of 1969, a major outbreak caused by a strain the previous winter, produces an immune community such that although the virus might be present during the summer, the usual winter outbreak is not evident. This possibility might be the case since there is usually a time lapse of two to three years between major epidemics of influenza and also since immunity to a strain is known to last for about three years.

Reports of isolations of the new Hong Kong strain point to the fact that data based on positive sera at 1:8 dilution and computed fortnightly can illustrate better the trend of outbreaks than data based on monthly results with the sample size used..

Effects of influenza A outbreaks on influenza B antibody observed in this study are similar to those reported by Widelock et al (1959) and Prezmycki et al (1959) that they noticed increases in post vaccination antibodies to B when A vaccines were used and also during an influenza A outbreak respectively. Our results show some degree of increase in influenza B antibody levels as influenza A antibody level increased.

The distribution of B antibody in the population is similar to that reported by Pereira (1967) from samples studied in England. This may be due to the fact that influenza B viruses have not changed that much over the years hence there is cross-reaction between the different strains.

Complement fixing anti-AS titres were found frequently to be lower than anti-AV and this substantiates the generally held view (Schmidt and Lennette 1965). From the results (Table XI) 45 out of 100 sera had equal anti-AS and anti-AV titres. In 47 sera anti-AV titres

were found to be higher than anti-AS and in 8 sera anti-AS was found to be higher than anti-AV. The results of the pooled sera also show that anti-AV titres are usually higher than anti-AS.

The relationship between humidity and incidence of influenza obtained in this survey was as expected.

Hemmes et al (1960) studied the relationship between influenza virus survival in air and seasonal variation of indoor humidity and reported that virus survival was optimal at the humidity obtained indoors during the winter. Their work relates to conditions obtained in North America where there is central heating system during the winter. Harper (1961) however showed that at low temperature and low humidity influenza virus survived better. His conditions are analogous to conditions in England and European countries where during the Winter the temperature and humidity indoors are nearly similar to those outdoors due to the poor system of heating. Two different conditions are therefore set by these workers for optimal virus survival. Influenza outbreaks however are known to occur during the winter seasons in many parts of the world with different winter conditions hence it appears that there is an interplay of humidity and temperature for virus survival.

The results of this survey indicate that high levels of positives to AS were obtained during the cold months suggesting high incidence of infection. During the winter the relative humidity indoors is low as a result of heating and as such virus survival is at its optimal and also personal contact indoors is increased since everybody is mostly indoors, hence the virus is easily passed around.

The CF microtechnique was found very suitable for this study for it allowed rapid titrations to be done and the use of small quantities of reagents. Zhdanov and Ritova (1959) reported that differences in avidity of influenza virus isolates suggest that HI may not be a sufficient index of the level of immunity of a population. Hobson and Pearson (1960) compared the HI and CF methods and reported that the recall phenomenom was not significantly evident in HI test hence only strains very closely related to the offending viruses could be used to achieve meaningful results in the HI test.

The CF test allows the use of "V" antigens derived from strains of preceding years and this gives diagnostically significant answers (Henle et al 1960).

Part II.

Persistence and Immunoglobulin Nature of anti "S" and anti "V" Complement Fixing Antibodies

Introduction: Complement fixing antibodies to the soluble and viral antigens of influenza viruses differ in their persistence in the blood. Anti "S" antibodies decline rapidly during convalescence to undetectable levels whereas anti "V" last for a longer time (Schmidt and Lennette 1965).

This part of the study was undertaken to examine this behaviour of anti "S" and anti "V" and to determine whether the relative transient nature of anti "S" could be explained on the basis that anti "S" and anti "V" complement fixing antibodies are made of different immunoglobulins.

Materials and Methods: Sera from two patients were collected over a period of time starting from the acute phase of infection. Serum samples were obtained for a period of one year in one case (9 collections) and over a period of 6 months (4 collections) in the other. These sera were stored at -20°C and were all titrated at the same time for CF titres by the micro-technique using Influenza A soluble and Influenza A2/Canada/9/66 antigens.

2-mercaptoethanol (2-ME) treatment was chosen for the immunoglobulin studies in view of the small volumes of sera available and the relative simplicity of this method.

Sera were treated with 1:10 volume of 0.5M 2-ME for one hour at 37°C (Banatvala et al 1967). Preliminary studies were done using sera from the population survey to find out whether there were any differences in titres between 2-ME treated sera, dialysed overnight against buffer (Stollar and Sandberg 1966) and titres of undialysed samples (Murray et al 1965). No difference was found using these two methods hence the experiment was performed without dialysis.

9 volumes of the serum was mixed with 1 volume of 0.5M. 2-ME and then placed at 37°C for one hour. Enough diluent was added to produce 1:4 dilution of the serum and this was inactivated at 56°C for 30 minutes. Titration was done in the usual manner by the CF microtechnique.

Results: Table XIV shows the results of the preliminary work with 2-mercaptoethanol (2-ME) using some of the sera from the population samples. Those treated samples which were not dialysed against buffer became slightly turbid when inactivated at 56°C for 30 minutes but these did not show any anti-complementary activity.

The effect of mercaptoethanol on sera collected from the two patients, AT and DP, is shown in the Table XV. It will be seen that in almost all the sera there was 50% reduction in titre. Fig. 15 and Table XV show CF antibody patterns in the two patients. Anti-AS and anti-AV peak levels were detected not later than the 10th day after infection. Anti-AS was maintained longer at peak level than anti-AV in patient AT whereas these antibodies were equally maintained and fell at the same rates in patient DP.

TABLE XIV

Results of Preliminary studies with 2-Mercaptoethanol (2-ME)

Serum No.	ANTIBODY TITRE									
	Anti AS					Anti AV				
	Normal CF	N	N/D	ME	ME/D	Normal CF	N	N/D	ME	ME/D
905	64	64	64	32	32	64	64	64	32	32
913	128	128	128	64	64	1024	1024	1024	512	512
977	32	32	32	16	16	256	256	256	128	128
991	32	32	32	16	16	64	64	64	32	32

Normal CF = Normal Complement fixation test

N = Native serum placed at 37°C for 1 hour.

N/D = Native serum placed at 37°C for 1 hour and dialysed overnight.

ME = 2-ME treated serum at 37°C for 1 hour.

ME/D = 2-ME treated serum at 37°C for 1 hour and dialysed overnight.

Table XV

2-Mercaptoethanol Treatment of Sera collected from Patients AT & DP

Serum No.	Date taken	CF Antibody Titre			
		Anti-AS		Anti-AV	
		Untreated	2-ME	Untreated	2-ME
AT 1	19/1/68	16	8	64	32
2	29/1/68	64	32	1024	512
3	29/2/68	64	32	1024	256
4	29/3/68	64	32	512	512
5	29/5/68	64	16	512	256
6	2/7/68	32	32	512	256
7	31/7/68	32	16	256	256
8	3/11/68	32	16	64	32
9	3/12/68	16	16	32	32
DP 1	30/1/68	< 8	< 8	< 8	< 8
2	7/2/68	64	64	128	128
3	29/3/68	64	32	128	64
4	21/6/68	32	32	64	64

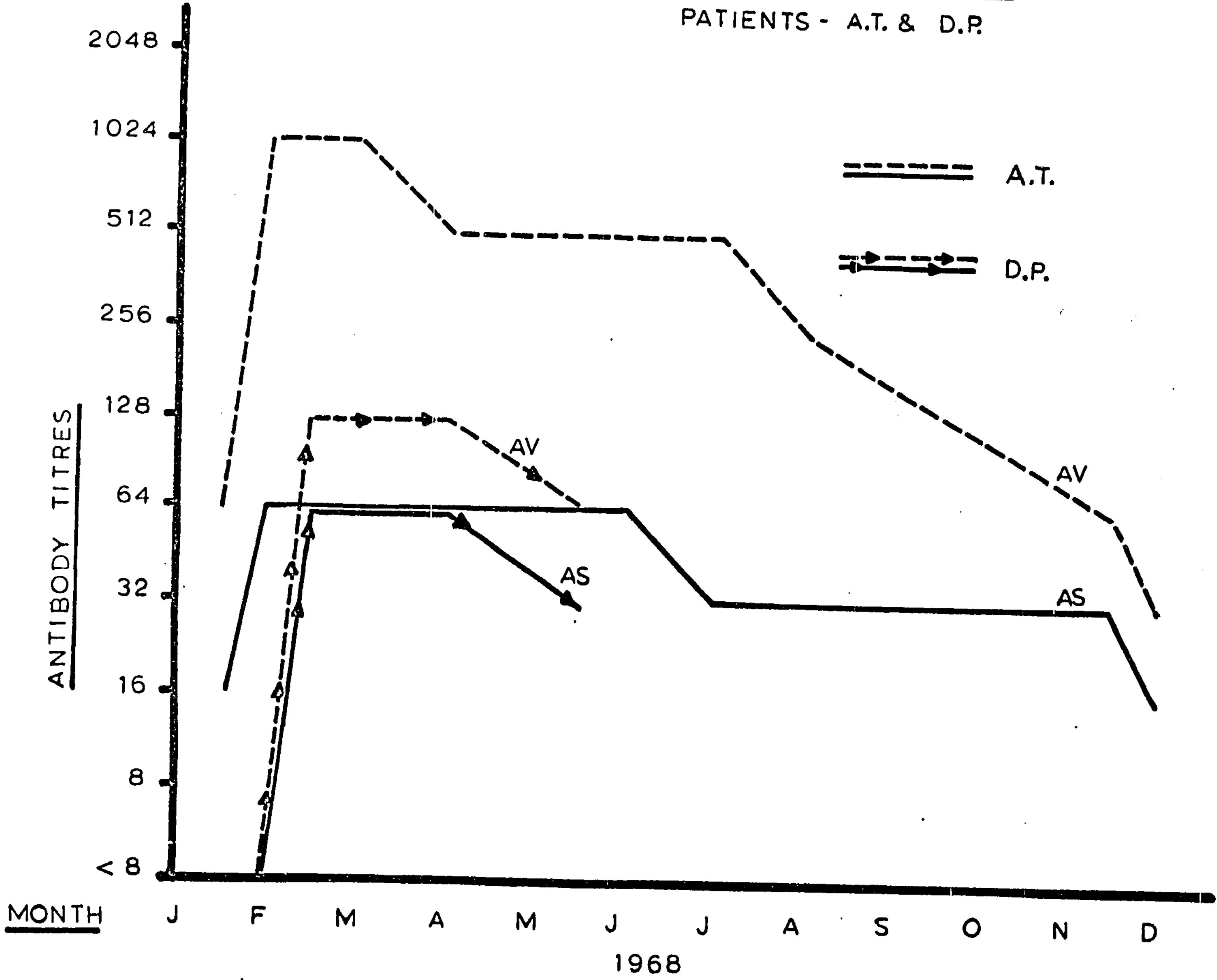
FIG. 15
C.F. ANTIBODY PATTERNS.
PATIENTS - A.T. & D.P.
A.T.
D.P.
ANTIBODY TITRES
2048
1024
512
256
128
64
32
16
8
< 8
AV
AS
AV
AS
MONTH
J
F
M
A
M
J
J
A
S
O
N
D
1968

Discussion: The number of individuals studied with regard to persistence of different CF antibodies did not permit positive conclusions to be drawn. However the results show that there was not much difference in rate of fall of anti-AS and anti-AV. Perhaps the disappearance of anti-AS could be explained by the fact that anti-AV titre is mostly higher than anti-AS hence at a certain time during convalescence only anti-AV will still be detectable.

Culver et al (1960) reported that CF antibodies reach peak levels at 9-10 days after vaccination and these are maintained for one month and in some cases for 2 or more months. In this study, peak levels of anti-AS and anti-AV were detected on the 7th day in patient DP and on the 10th day in patient AT. No samples were collected between the first and 7th day in DP and between the first and 10th day in AT. In one instance both anti-AS and anti-AV were detected at the end of the one year period.

It was assumed in the immunoglobulin studies that antibodies sensitive to a disulphide bond reducing agent are 19S antibodies. Other investigators have made a similar assumption and found no inconsistencies when

this method is cross-checked with sucrose gradient ultracentrifugation and immunoelectrophoresis (Uhr and Finkelstein 1963; Svehag and Mandel 1964).

Wiedermann et al (1963) reported that treatment of 7S immune globulins with a sulphydryl reagents alters the ability to fix complement after combining with antigen. Murray et al (1965) however reported that exposure of serum to 0.03 M 2-ME without subsequent alkylation as done by Widermann et al, had no effect on complement fixation by 7S antibody while it did abolish the activity of 19S antibodies. At 0.1 M concentration complement fixation activity of 7S antibodies was moderately reduced. There was no reduction in activity if concentration of 2-ME was kept between the limits of 0.06 to 0.03 M.

In this experiment 0.05M final concentration was used.

Murray et al (1965) reported the use of 2-ME treatment to differentiate 19S and 7S CF. antibodies in primary and recrudescent Typhus infection. They reported 95% reduction in 19S activity. Stollar and Sandberg (1966) used 0.1 M 2-ME to characterise 19S and 7S antibodies after separation of these antibodies by

column chromatography using Sephadex G200. They reported more than 95% loss of CF activity of 19S and only 25% loss in 7S antibody. Other workers have reported similar results and have found mercaptoethanol treatment suitable for distinguishing 19S and 7S antibodies. (Sandberg and Stollar 1966; Berlin 1966; Bantvala et al 1967).

Berlin (1966) reported that when mice were injected intraperitoneally with emulsified influenza virus vaccine, γM antibody accounted for most of the HI. activity through day seven, but γG type antibody predominated after that, as shown by sedimentation by sucrose gradient with ultracentrifugation and treatment with 2-ME. Waldman et al (1967) examined the sera of human adult volunteers who were immunized parentally with dead influenza virus and reported that the neutralizing antibody formed belonged solely to the IgG class of immunoglobulin. Their previous work had shown that the same statement was true of the antibody formed after experimental infection with influenza A2 virus administered via the upper respiratory tract.

No report is available on the immunoglobulin nature of the complement fixing (CF) antibodies to

influenza.

The results of our studies suggest that both anti "S" and anti "V" CF antibodies consist of 19S and 7S antibodies respectively if the contention of Murray et al about the critical level of 2-ME is accepted and that the difference in anti "S" and anti "V" persistence is not due to immunoglobulin differences.

General Summary

3,400 sera were examined by the CF microtechnique and from information obtained, a new approach is suggested for the prediction of epidemics.

Results indicated that anti-AS CF titres are usually lower than anti-AV titres; there was considerable cross-reaction between influenza A2 viruses used and A2/Hong Kong/68 was found to be more related to A2/Singapore/57 than to A2/Canada/66.

Influenza outbreaks in 1967-68 and 1968-69 were caused by influenza A and these seemed to have effect on influenza B antibody levels. A summer prevalence of Influenza A was noticed in 1968.

CF antibodies to the soluble and viral antigens were both found to contain mercaptoethanol sensitive as well as mercaptoethanol resistant immunoglobulins.

ADDENDUM

THE HONG KONG 'FLU EPIDEMIC

CURRENT SITUATION

Influenza A2 showed no change until mid July 1969 when a new strain was noticed in an extensive epidemic in Hong Kong. The disease spread rapidly to Singapore, Malaysia and Australia. In the autumn the spread of the infection appeared to have slowed down and epidemics were less widely distributed than was originally expected. The U.S. was the only country to report high incidence by the end of 1968 (W.H.O. Chronicle, 1969, 23, 362).

Scattered outbreaks of influenza-like illness were reported in Canada, especially in the Western Provinces, by the end of December 1968. In the Central Provinces, the only activity of note was in Montreal while the Maritime Provinces appeared to be virtually free of the disease. Isolations were however made in some of the Atlantic Provinces in January (Epidemological Bulletin, 1968 (12)) and by the end of January, A2/Hong Kong had been isolated in all the provinces of Canada. The peak of influenza

A2/Hong Kong activity in North America occurred in early January 1969 and declined rapidly (W.H.O. Report, April 1969). Since late February very little influenza A2 activity has been reported. The epidemics were regarded as extensive in the U.S.

Immunological studies on populations in most parts of the world indicated that most of the sera taken before the appearance of A2/Hong Kong, showed no antibody or negligible HI titres ranging from 59% negative sera in Budapest to 98% negative sera in West Berlin and that most of the positive sera belonged to the older age groups. (W.H.O. Report, April 1969) Serological survey of human sera selected from various regions of Ontario however showed the presence of antibodies to the Hong Kong strains in an appreciable number of individuals (Zalan and Labzoffsky, 1969).

A striking feature of the present situation of influenza is that in every country where the Hong Kong/68 variant has been detected, it has completely replaced former variants. (W.H.O. Report, April 1969)

U.S. Morbidity and Mortality Report (July 1969) indicated that progressive increases in the incidence of A2/Hong Kong were being reported in South America and in many countries in the Southern Hemisphere by June and that there was a possibility of a second

incidence of Hong Kong 'flu in North America in the winter of 1969.

REFERENCES

1. Epidemiological Bulletin. (by Dept. of National Health and Welfare, Ottawa) December 1968, 12 (12).

2. Morbidity and Mortality Report. July 1969 by U.S. Dept. of Health, Education and Welfare.

3. W.H.O. Chronicle. August 1969, 23, 362.

4. W.H.O. Report of Meeting of Directors of Virus Reference Centres. April 1969.

5. ZALAN, E., and LABZOFFSKY, N.A. (1969). Antigenic Aspects of Influenza A2 strains isolated in Ontario. Canad. J. Public Health. 60, 32.

APPENDIX

(1) * DILUENT (Veronal Buffered Saline)
STOCK SOLUTION

NaCl .... 85 Gm

Barbituric acid...... 5.75Gm.

Dissolve the barbituric acid in 500 mI hot distilled water. Add the NaCl and make up volume to 1500 ml.

b) Dissolve sodium barbiturate 1.25Gm in 500 ml. distilled water and add to the NaCl/barbituric acid solution until pH is 7.2.

Make up to a final volume of 2000ml then add

$MgCl_2$ $6H_2O$ ....1.68Gm.

$CaCl_2$ ....0.28Gm.

Filter the solution and store at 4^0C. This gives five times (5X) concentration of diluent. Dilute 1 in 5 with distilled water before use.

(2) ANTIGEN TITRATION

CHESS BOARD METHOD

Seven tubes are set up in a row for antigen dilution and another nine tubes for antiserum dilutions. Two fold dilutions of antigen and antiserum are made

* After Virus Reference Laboratory, Colindale, London.

using diluent. One disposable plate is taken and marked as in the diagram overleaf. Starting with the antigen dilutions, 0.025 ml. is delivered from the 1:2 sample into the cups in row 1 downwards i.e. from A to J. This procedure is repeated for all the antigen dilution. New pipettes are used for every dilution. Row 8 receives 0.025 ml diluent.

The antiserum dilutions are next added starting with the lowest dilution from row A across to cup No.8. Row J receives 0.025 ml diluent - 0.025 ml of 1:30 complement solution is added to each cup and the plate is incubated at 4°C for 18 hours (overnight). The plate is removed and kept at room temperature for 10 minutes. 0.025 ml sensitized sheep cells is added to each cup and the plate is incubated at 37°C for 30 minutes, placed at 4°C for about 2 hours and results read.

Optimal dilution of antigen is the dilution which fixes not less than 75% complement with the highest dilution of antiserum. Optimal dilution of each antigen is used in the test proper.

Diagram of plate markings for titration

Antigen dilutions

Cup No.	1	2	3	4	5	6	7	8
Dilution 1 in	2	4	8	16	32	64	128	antiserum controls.

Cup No;	
A	2
B	4
C	8
D	16
E	32
F	64
G	128
H	256
I	512
J	Antigen controls

(3) ALSEVER'S SOLUTION

Dextrose ....2.05 Gm.

Trisodium citrate
(dihydrate) ....0.8 Gm.

Sodium chloride ....0.42 Gm.

Dissolve in 100 ml distilled water. This solution is adjusted to pH. 6.1 with 10% citric acid and sterilized by filtration.

(4) Sheep Red blood cells: Red blood cells are collected in equal volume of sterile 2.5% sodium citrate or Alsever's solution and centrifuged. The sediment is washed three times with Alsever's solution and stored in solution at 2°C to 4°C.

(5) COMPLEMENT TITRATION

(i) Dilution

Tube Number	Complement (1:10) ml.	diluent	Dilution
1	0.5	2ml	1:50
2	0.5	2.5ml	1:60
3	0.5	3.0ml	1:70
4	0.5	3.5ml	1:80
5	0.25	2.0ml	1:90
6	0.25	2.25ml	1:100
7	0.25	3.5ml	1:150
8	0.25	4.75ml	1:200
9	0.10	2.4ml	1:250
10	0.10	2.9ml	1:300

(ii) Titration: Incubate 0.2ml of each dilution of complement with 0.2ml of optimal dilution of each antigen and 0.2 ml diluent (representing antiserum) at 4°C overnight. Add o.2 ml haemolytic

system (sensitized sheep cells) and incubate at 37°C for 30 minutes. Place at 4°C for about 2 hours. Read results.

1 MHD is the highest dilution of complement showing 100% haemolysis.

3 MHD of complement was in the experiment.

REFERENCES

1. ANDREWES, C.H., (1967)
Influenza, Ringer of Changes, in the Natural History of Viruses; Weindenfeld and Nicolson London (pp. 54-67)

2. BANATVALA, J.E., Best, J., Kennedy, E.A., Smith, E.E., and Spence, M.E. (1967)
Serological method of demonstrating recent infection by Rubella Virus
Brit. Med. J., 3; 285

3. BERLIN, B.S., (1966)
Early antibody responses of mice given a single injection of emulsified Influenza Virus Vaccine.
J. Immunol. 96; 424

4. Committee on Standard Serological Procedures in Influenza stúdies (1950)
J. Immunol. 65; 347

5. COUCH, R.B., Douglas, R.G., Kasel, J., Riggs, S., and Knight, V., (1969)
Production of the Influenzal syndrome in man with Equine Influenza Virus
Abstract - Bacteriological Proceedings, American Society for Microbiology

6. CULVER, J.O., Lennette, E.H., Navarre, G., and Kempe, C.H., (1960)
The response to Influenza Vaccines as Determined by levels of Complement fixing Antibody in the Acute-phase sera of Patients with Respiratory Disease.
J. Immunol. 84; 98

7. DAVENPORT, F.M., Hennessy, A.V., and Francis, T., Jr. (1953)
Epidemiologic and immunologic significance of age, distribution of antibody to antigenic variants of influenza virus.
J. Exp. Med. 98; 641

8. DAVENPORT, F.M., and Hennessy, A.V. (1958)
The clinical epidemiology of Asian influenza.
Ann. intern. Med., 49; 493

9. DAVENPORT, F.M., and Minuse E., (1964)
Influenza Viruses, Chapter 13, In Diagnostic Procedures for viral and Rickettsial Diseases, 3rd Edition, Am. Public Health Ass. Inc.

10. DEMPSTER, G., (1968)
Influenza: Clinical, Epidemiologic and Immunologic Features, In Textbook of Virology (A.J. Rhodes and C.E. van Rooyen) 5th Edition. (pp.429-451)

11. DEPOUX, R., Orio, J., Chippaux, C., Merville, P., (1959)
La grippe dans la population Africaine de Brazzaville.
Bull. Org. mond Santé, 20; 241

12. DUNN, F.L., (1958)
Pandemic Influenza in 1957. Review of International spread of new Asian strain.
J. Amer. med. Ass. 166; 1140

13. FABIYI, A., Lief, F.S., and Henle W., (1958)
Antigenic analysis of influenza viruses by complement-fixation.
II The production of antisera to strain specific "V" antigens in guinea pigs.
J. Immunol. 81; 467

14. FAZEKAS DE St. GROTH, S., and Donnelley, M., (1950a)
Studies in experimental immunology of influenza (iii) The antibody response.
Austral. J. exp. Biol. med. Science. 28; 45

15. ______________________________, (1950b)
Studies in experimental immunology of influenza (iv) The protective value of active immunization.
Austral. J. exp. Biol. med. Science 28; 61

16. FRANCIS, T., Jr., Pearson, H.E., Salk, J.E., and Brown, P.N. (1944)
Immunity in human subjects artificially infected with influenza virus Type B.
Am. J. Pub. Health. 34; 317.

17. FRANCIS, T., Jr., (1953)
Vaccination against influenza.
Bull. Wld. Hlth. Org. 8; 725

18. FUKUMI, H., (1959)

Human vaccination experiments with Asian influenza vaccine in Japan.
Bull. Wld. Hlth. Org., 20; 355.

19. FULTON, F., and Dumbell, K.R., (1949)
The serological comparison of strains of influenza virus.
J. Gen. Microbiol. 3; 97

20. FYSON, R., and Westwood, J.C.N. (1969)
unpublished.

21. HALE, J.H. (1961)
Duration of immunity in virus diseases
Advances in Immunology 1; 263

22. HARPER, G.J., (1961)
Airborne micro-organisms: survival test with four viruses.
J. Hyg., Camb. 59; 479

23. HEMMES, J.H., Winkler, K.C., and Kool, S.M., (1960)
Virus survival as a seasonal factor in influenza and poliomyelitis.
Nature 188; 430

24. HENLE, G., Harris, G., and Henle W. (1948)
The reactivity of various human sera with mumps complement fixation antigens.
J. Exptl. med. 88; 133

25. HENLE, W., and Lief, F.S., (1960)
Antigenic analysis of influenza viruses by complement fixation
(iv) Strain - specific V. antigens in serodiagnosis of influenza.
J. Immunol. 84., 491

26. HILLEMAN, M.R., Flatley, F.J. Anderson, S.A., Luecking, M.L., and Levinson, D.J., (1958)
Distribution and significance of Asian and other influenza antibody in the human population.
The New Eng. J. of med., Vol. 258, 20; 969

27. HOBSON, D., and Pearson, E., (1961)
Serological responses to Asian influenza viruses in man: A comparison of Haemagglutination inhibition and complement fixation methods.
Brit. J. Exp. Path. 42; 53

28. HOBSON, D., Lane, C.A., Beare, A.S., and Chivers, C.P., (1964)
Serological studies on adult volunteers inoculated with oil-adjuvant Asian influenza vaccine. Report to the M.R.C. Committee on influenza and respiratory virus vaccines.
Brit. med. J. 2; 271

29. HORSFALL, F.L., Jr., and Tamm I., (1965)
Viral and Rickettsial Infections of Man 4th Edition (Lippincott Co.)

30. HOYLE, L., (1945)
An analysis of the CFT reaction in influenza
J. Hygiene 44; 170

31. ISAACS, A., (1957)
Antibody to influenza viruses in the sera of Nigerians.
J. Hygiene (Camb.) 55; 484

32. ISAACS, A., Hart, R.J., and Law V.G. (1962)
Influenza viruses 1957-1960.
Bull. Wld. Hlth. Org. 26; 253.

33. JAWETZ, E., Melnick, J.L. and Adelberg, E.A., (1962)
Viral diseases of the respiratory tract and parotid glands. Chap. 13 in Review of Medical Microbiology 5th Edition.

34. JENSEN, K.E., Dunn, F.L., and Robinson, R.Q., (1958)
Influenza 1957. A variant and the pandemic.
Progr. med. Virol. 1; 165

35. JOHNSON, C.M., and Westwood, J.C.N., (1968)
Immunodiffusion studies on the myxoviruses, I. The presence of influenza A/PR8 precipitins in the serum of some normal animals.
Canadian Journal of Microbiology 14; 53

36. ______________________, (1969) unpublished.

37. KAPLAN, M.M., and Payne A. M-M., (1959)
Serological survey in animals for type A influenza in relation to the 1957 pandemic.
Bull. Wld. Hlth. Org. 20; 465

38. KILBOURNE, E.D., (1968)
Recombination of influenza A viruses of human and animal origin.
Science 160; 74

39. KIRBER, M.W., and Henle W., (1950)
A comparison of influenza complement fixation antigens derived from allantoic fluids and membranes.
J. Immunol. 65; 229

40. KWAPINSKI, J.B., (1965)
Methods of Serological Research.
John Wiley and Sons Inc. (pp. 276-314)

41. Leading Articles - Influenza Epidemic Brit. Med. J., 1968, 1; 3

42. LEHRICH, J.R., Kasel, J.A. and Rossen, R.D. (1966)
Immunoglobulin classes of neutralizing antibody formed after human inoculation with soluble adenoviral antigens.
J. Immunol. 97; 5

43. LIEF, F.S. and Henle W., (1959)
Methods and procedures for use of complement fixation technique in type and strain specific diagnosis of influenza
Bull. Wld. Hlth. Org. 20; 411

44. MAGILL, T.P., (1955)
Propagation of influenza virus in "immune" environments.
J. Expt. Med. 102; 279

45. Medical Research Council: Clinical trials of oil-adjuvant influenza vaccines 1960-3.
Report to the Medical Research Council by its Committee on influenza and other respiratory virus vaccines.
Brit. Med. J., 2; 267

46. MULDER, J., and Masurel, N., (1958)
Pre-epidemic antibody against 1957 strain of Asiatic influenza in serum of older people living in the Netherlands.
Lancet 1; 810

47. MASUREL, N., (1969)
Relationship between Hong Kong and former human isolates and A/Equi 2 virus in human sera collected before 1957.
Lancet 1; 907

48. MURRAY, E.S., O'Connor, J.M., and Gaon J.A., (1965)
Differentiation of 19S and 7S complement fixing antibodies in primary versus recrudescent Typhus by either eithanethiol or heat.
Proc. Soc. Exp. Biol. Med. 119; 291

49. PAYNE, A. M-M (1958)
Some aspects of the epidemiology of the 1957 influenza pandemic.
Proc. roy. Soc. Med. 51; 1009

50. PEREIRA, H.G., and Pereira, M.S., (1964)
Antigenic variants of influenza A2 virus.
Bull. Wld. Hlth. Org. 31; 129

51. PEREIRA, H.G., Tumova, B., and Law, V.G. (1965)
Avian influenza A viruses.
Bull. Wld. Hlth. Org. 32; 855

52. PEREIRA, M.S.,Chakraverty, P., and Pollock, T.M. (1967)
Survey of influenza antibody in England by the strain specific complement fixation test.
Brit. Med. J. 4; 80

53. PEREIRA, H.G., Tumova, B. and Webster, R.G., (1967)
Antigenic relationship between influenza A viruses of human and avian origins.
Nature 215; 982

54. PEREIRA, M.S., Chakraverty P., and McDonald, J.C. (1968)
The acquisition and persistence of antibody after infection with influenza A2 virus. A long term study of 25 families.
Personal communication.

55. PHIPPS, P.H., and Westwood, J.C.N. (1967) unpublished.

56. PICKLES, W.H., Burnet, F.M., and McArthur, M. (1947)
Epidemic respiratory infection in a rural population.
J. Hyg. (London) 45; 469

57. PRIER, J.E., Satz, J.E., Deardorff, J., and Schrack, W.D., Jr., (1969)
Early detection of the Hong Kong influenza epidemic by a respiratory virus surveillance programme.
Abstract - Bacteriological Proceedings.

58. PRZESMYCKI, F., Sawicki, L., and Dobrowolska, H., (1959)
Vaccination against influenza in Poland 1953 - 56.
Bull. Wld. Hlth. Org. 20; 333

59. RHODES, A.J., and Van Rooyen C.E., (1968)
Textbook of Virology. 5th Edition.
(pp. 417-418) William and Wilkin Co.
Baltimore.

60. ROBINSON, R.Q., (1964)
Natural history of influenza since the introduction of the A2 strain.
Prog. Med. Virol. 6; 82

61. SANDBERG, A.L., and Stollar D.B. (1966)
Comparisons of antibodies reacting with DNA. II: Rabbit antibodies induced by nucleoside-protein conjugates.
J. Immunol. 96; 764

62. SATZ, J.E., Prier, J.E., and Riley, R., (1969)
Characteristics of Hong Kong influenza virus.
Abstracts - Bacteriological Proceedings
American Society for Microbiology.

63. SCHILD, G.C., and Stuart-Harris C.H., (1967)
Antibodies in children's sera for serologically different recently isolated strains of influenza A2 and B.
Birt. med. J., 4; 82

64. SCHMIDT, N.J., and Lennette, E.H. (1965)
Basic Technics for Virology in Viral and Reckettsial Infections of Man.
4th Edition (Horsfall and Tamm)

65. SEVER, J.L., (1962)
Application of a microtechnique to viral serological investigations.
J. Immunol. 88; 320

66. SHOPE, R.E., (1931)
Swine influenza III Filtration experiments and etiology.
J. Exp. Med. 54; 373

67. ________________, (1938)
Serological evidence for the occurence of infection with human influenza virus in swine
J. Exp. Med. 67; 739

68. SIMPSON, R.W., (1969)
Influenza virus: Genetics and Control
Science 163; 409

69. SLEPUSHKIN, A.N. (1959)
The effect of a previous attach of A1 influenza on susceptibility to A2 virus during the 1957 outbreak
Bull. Wld. Hlth. Org. 20; 297

70. SMITH, W.S., Andrewes, C.H., and Laidlaw P.P., (1933)
A virus obtained from influenza patients.
Lancet 2; 66

71. STALLONES, R.A., and Lennette E.H., (1959)
Epidemiologic observations of an outbreak of Asian-strain influenza in a closed population.
Am. J. Pub. Health. 49; 656

72. STOLLAR, B.D., and Sandberg, A.L., (1966)
Comparisons of Antibodies Reacting with DNA. (i) Systemic Lupus erythematosus sera and rabbit antibodies induced by DNA -methylated Bovine serum albumin complexes.
J. Immunol. 96; 755

73. STUART-HARRIS, C.H., (1965)
Influenza and other virus infections of the respiratory tract. 2nd Edition.
Edward Arnold (Publishers) Ltd. London

74. SVEHAG, S., and Mandel, B., (1964)
The formation and properties of poliovirus neutralizing antibodies. II. 19S and 7S antibody dose requirement for sustained synthesis, anamnesis and sensitivity to X-irradiation
J. Exp: Med. 119; 21

75. TAYLOR-ROBINSON, D., (1965)
Respiratory virus antibodies in human sera from different regions of the world.
Bull. Wld. Hlth. Org. 32; 833

76. TYRRELL, D.A.J., (1967)
Common colds and related diseases.
Brit. med. Bull. 23; 2

77. TUMOVA, B., and Pereira, H.G., (1965)
Genetic interaction between influenza
A viruses of human and animal origin
Virology 27; 253

78. UHR, J.W., and Finkelstein, M.S., (1963)
Antibody formation (IV) Formation of
rapidly and slowly sedimenting antibody
and immunological memory to bacterio-
phage ØX174.
J. Exp. Med. 117; 457

79. WALDMAN, R.H., Kasel, J.A., Alford, R.H.,
and Mann, J.L., (1967)
Immunoglobulin classes of serum neutrali-
zing antibody formed in response to
infection with human A2 influenza virus.
Proc. Soc. Expt. Biol. Med. 125; 316

80. WALDMAN, R.H., Mann, J.J., and Kasel,
J.A., (1967)
Immunoglobulin classes of serum neutrali-
zing antibody formed in response to
immunization with dead influenza virus
vaccine.
Proc. Soc. Exp. Bio. Med. 126; 838

81. WALLACE, G.D., and Kissling, R.E. (1959)
Studies in swine of Asian influenza virus.
Bull. Wld. Hlth. Org. 20; 455

82. WEBSTER, R.G., and Laver, W.G., (1966)
Influenza virus submit vaccines: Immuno-
genicity and lack of Toxicity for rabbit
of ether and detergent - disrupted virus.
J. Immunol. 96; 596

83. WIDELOCK, D., Klein, S., Simonovic, O.,
and Peizer L.R. (1959)
A laboratory analysis of the 1957-1958
influenza outbreak in New York City.
II. A sero-epidemiological study
Am. J. Pub. Health 49; 847

84. WIEDERMANN, G., Miescher, P.A., and
Franklin, E.G., (1963)
Effect of mercaptoethanol on comple-
ment binding ability of human 7S
gammaglobulin.
Proc. Soc. Exp. Biol. Med. 113; 609

85. WILSON, G.S., and Miles, A.A. (1964)
Topley and Wilson's Principles of Bacteriology and Immunity. 5th Edition
Vol. 2; 1989

86. W.H.O. (1959), Technical report series No. 170: Expert Committee on Respiratory Disease.

87. ZALAN E., and Labsoffsky, N.A., (1969)
Influenza A2 strains isolated in Ontario: Antigemic comparison with classical Asian and Hong Kong strains.
Canad. Med. Ass. J. 100 No. 19; 901

88. ZHDANOV, V.M., (1959)
Results of further research on influenza in the USSR with special reference to the 1957 pandemic.
Bull. Wld. Hlth. Org. 20; 261

89. ZHDANOV, V.M., and Ritova, V.V., (1959)
Quelques particularités cliniques et sérologiques de la grippe en 1957/58 chez des enfants.
Bull. Org. mond. Santé, 20; 251

90. ZHDANOV, V.M., (1967)
The 1967 outbreak of influenza B in the USSR.
Lancet 1; 263