STUDIES ON INACTIVATED INFLUENZA VACCINES

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JOCELYN ROBERT LANE FORSYTH

M.B., Ch.B., Dip. Bact.

Department of Microbiology

The Queen's University of Belfast

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CONTENTS

PREFACE

It is, perhaps, ironical that while influenza was probably the first animal virus to be studied in detail there is still little known concerning its behaviour. In many ways it represents a model of difficult immunization problems. Now that so many of the most lethal and dramatic of the infectious diseases are susceptible to control attention is being focused upon the control of the next rank of disorders. This includes those conditions which cause ill-health and discomfort only to such an extent that the cost and severity of measures of prevention must be carefully weighed against the effects of the disease process itself. The solution of the technical, administrative and ethical problems of influenza control will, in addition, greatly assist the struggle against numerous other ailments.

CHAPTER I

HISTORICAL INTRODUCTION

THE STORY OF INFLUENZA:

It would seem that influenza has long been with mankind. Edmund Parkes (1866), in Reynolds' "A System of Medicine", attributes its description to Hippocrates and discerns influenza in the malady of the ill-fated Athenian expedition to Sicily. August Hirsch (1883) cited epidemics as far back as 1173 that tallied with influenza.

In the wealth of description the clinical picture of influenza has remained constant. It is not surprising that the quotation by Thomas Short (1749, cited by Thompson, 1852) of a description of an outbreak in Madrid in 1557 has figured so much in the literature for it keeps its lucidity to-day. "There it began with a roughness of the jaws, small cough, then a strong fever, with a pain of the head, back, and legs; some felt as though they were corded over the breast, and had a weight at the stomach, all of which continued to the third day at farthest; then the fever went off with a sweat, or bleeding at the nose. In some few, it turned to a pleurisy, or fatal peripneumony."

The epidemic nature of influenza has always been one of the most striking features of the disease. Hirsch

wrote that in its pandemic character "....influenza takes an exceptional place among the acute infective diseases". Many of the early names expressed the concept of novelty as in 1558 the "new burning ague" or in 1562 the account from the court of Mary Queen of Scots of "the newe acquaytance".

With these recurring epidemics went a pecubiarly high attack rate and these combined to impress the chroniclers of the disease. "It attacked at once, and raged all over Europe, not missing a family, and scarce a person" writes Short (1749) of the outbreak of 1510. Huxham (1758, cited by Thompson, 1852) reflected upon 1733, "About this time a disease invaded these parts, which was the most compleatly epidemic of any I remember to have met with; not a house was free of it; the beggar's hut, and the nobleman's palace were alike subject to its attacks; scarce a person escaping either in town or country; old and young, strong and infirm, shared the same fate." The Royal College of Physicians in London reported in 1783, cited by Thompson (1852): "The universality of the influenza was remarkable, the proportion of the inhabitants affected by it being in some places estimated at three-fourths, in other places, at four-fifths of the whole".

Despite the numerous cases the mortality was recognized to be generally low. Huxham continued his

account mentioned above "....but still considering the great multitude that were seized by it it was fatal to but few, and that chiefly infants and consumptive old people". The Ambassador in Scotland to Queen Elizabeth the First wrote that "There was no appearance of danger, nor manie that die of the disease, excepte some old folks" (Brit. Med. J., 1919).

THE NATURE OF EPIDEMICS:

In the last century with the greater organisation and enumeration of populations the spread of epidemics has been more readily established. It has become clear that world-wide pandemics occur at fairly long intervals of 10-20 years but in between these major waves, which are associated usually with variation of the virus, come more localised outbreaks restricted to a country, a province, a town or even a community.

Certain general predictions may be made in that influenza epidemics tend to avoid the summer. A pandemic will tend to rage in the winter in one hemisphere and invade the other with the change of season. In the tropics the seasons have much less influence.

However, even a superficial study of the major recent pandemics shows a marked variation in behaviour for reasons at present unknown.

Before the era of viral identification, the pandemic of 1918-1919 presents an extraordinary picture. Influenza was reported in April 1917 (Ireland, 1928) in the United States. A high incidence of a relatively mild disease occurred during the winter of 1917-1918, in the northern hemisphere, rising to a peak in April 1918. This then fell to a low level in June 1918. At this time the disease changed its character on both sides of the Atlantic simultaneously and in the following months the immensely lethal epidemic raged despite the summer season and spread about the world.

In 1946 a new "family" of viruses was ushered in quietly in Australia. These spread across the Pacific and invaded the United States in the northern winter and spring. However, Britain was, relatively, spared in that winter and, with continental Europe, was much more affected the following winter when an epidemic sprang from a multicentric source in Sardinia. The most severe wave in Britain of the same family of viruses occurred in 1950-1951. Thus the pandemic phase of this variant was of a much more insidious nature.

In contrast, the Asian pandemic of 1957 spread across the world in text-book fashion, affecting China and Japan in the winter and spring, causing many cases in South America and South Africa in the southern winter and

creating widespread epidemics in the northern hemisphere in the autumn.

Frequently, during these pandemic phases it was possible to trace the spread of infection along the lines of communication - of ships from New Zealand to Fiji in 1918 (Thomson and Thomson, 1934), of aircraft from the Far East and Hawaii to California in 1947 (Sartwell and Long, 1947). It is also possible to see examples of that explosive spread, faster than travel, which so puzzled older epidemiologists and led them to feel that influenza was not spread by human contact (Parkes, 1866). This was the effect of the "seeding"hypothesis of Andrewes (Andrewes, 1953) well shown in the United States in 1957 (Langmuir, 1961) and in numerous lesser outbreaks before and since (Forsyth, 1962).

While it is possible to see a mechanism for pandemic spread operating in the phenomenon of antigenic variation, it is, as yet, impossible to do more than speculate on the significance of the wider epidemiological oscillation shown by Stuart-Harris (1953 - pp. 95-98). Here the evidence is put forward for a period of 50 years, from 1847 to 1889, without influenza which has been followed by our present cycle of epidemics. We cannot know the cause of this or the relation of it to our present observations.

INFLUENZAL MORBIDITY:

In Thompson's "Annals of Influenza" (1852) various authors give their opinions on the age distribution of influenza but their accounts are conflicting. Sydenham in 1675 remarked "No one escaped then, whatever might be his age or temperament". The Royal College of Physicians of London, quoted before, felt "....it was by no means partial to any age, sex or temperament. In London, however, it was observed to affect a much smaller proportion of children than of adults". Molyneux, in Dublin, however, reported of the influenza of 1693 that "....it rather favoured the very old, who seldom were attacked with it".

However, it is only in this century that more detailed and reliable evidence of age-specific attack rates have become available. The data from the United States have been analyzed by Collins (1944, 1957). Although differences may occur in different epidemics an overall pattern exists.

Rising from a high level in infancy the maximum incidence occurs in the age group 5-9. From here there is a fall to a lower figure lying between the ages of 15 and 25. This is followed by a slight rise to 30-35 with a falling incidence with advancing age (Francis, 1953). The most constant features are the low attack rate in old people and the high rate in young school-children. In the

Asian epidemic of 1957 the rather older school-children appeared to be affected more (Langmuir, 1961, Fry, 1957).

This distribution is probably the resultant of the two factors of resistance and exposure and the weight of each factor is still controversial. The theories of Francis and associates, to be described below, place much weight upon resistance, arguing that it is the antibody spectrum of older people that accounts for their low influenza attack rate.

However, it could be argued that the high level of infection in school-children is, at least in part, associated with the increased exposure to which they are subjected and that the schools may act as epidemic "detonators", so to speak.

Thomson and Thomson (1934) review the opinions expressed after the 1918-1919 pandemic and these are fairly divided, with some feeling that the schools are important and that closing the schools is associated with a reduction in the epidemic, while others feel that schools are little concerned in the spread of influenza - E.O. Jordan (1927), in his book, inclines to the latter view. Hope-Simpson, in a discussion at the Royal Society of Medicine (1951), felt that his study on the 1951 epidemic in Britain showed that influenza, unlike measles, is not brought home from school to the family. However, when W.S. Jordan (Jordan, Denny,

Badger, Curtiss, Dingle, Oseasohn, and Stevens, 1958, Jordan, 1961) reviewed the mechanisms of spread of Asian influenza he noted that in Great Britain, Japan and the United States outbreaks occurred primarily among school-children and then spread to younger children and adults. The times of the outbreaks were related to the dates of the school terms in a positive manner. The family study in Cleveland showed that school-children brought the infection into the home in 83% of family episodes. This interpretation is supported by Philip, Bell, Davis, Beem and Beigelman (1961) who, over five years, carried out a community study of influenza. These workers showed that "school-age children comprised the most important link in the chain of infection between the family and the community". This concept is supported by Chin (Chin, Mosley, Poland, Rush, Belden and Johnson, 1963) in his studies during outbreaks of influenza B.

It would seem that the most comprehensive and most careful studies appear to favour the importance of schools as epidemic centres.

Whereas Davenport and Hennessy (1960) have pointed out that an influenza epidemic is not a light matter to children, a community is most severely affected by the morbidity among the working population with the subsequent financial loss.

Norwood and Sachs (1947) stress that respiratory infections head the list of medical causes for employee absenteeism and that epidemics of influenza may quickly incapacitate so many employees as to make the operation of a plant extremely difficult or impossible. In an era of increasing productivity fewer people are responsible for more output and the effect of an attack rate of 20% is extremely serious (Shephard, 1961). McDonald (1963) estimated that 50 days of lost time per 100 people per annum throughout Western Europe were caused by influenza from 1955-1959. The effect of the Asian epidemic on absence from work due to sickness was described as "drastic" (Fortuin, Soeters and Van Beek, 1962). Fry (1957) calculates that in his practice of 5,500 people, 4,000 working days were lost in this epidemic.

The cost of influenza in industry is felt by the employer in absenteeism causing loss of production and increased costs in overtime rates and by the community in loss of wages and in sickness benefits paid out. Roden (1963) points out that in September through November, 1957, new sickness benefit claims rose to 1½ million above the usual level. Less susceptible to calculation is the effect of post-influenzal depression, which Stuart-Harris felt was a well recognised sequel to influenza (Stuart-Harris, 1961).

INFLUENZAL MORTALITY:

It has been mentioned above that the mortality rate of influenza is low. However, in view of the high attack rate the actual incidence of deaths complicating influenza is by no means insignificant. The Public Health Laboratory Service of England and Wales estimated that during September, October, November, 1957, there were 16,000 excess deaths in England and Wales although the case fatality was no higher than 1:1000 (Public Health Laboratory Service Report, 1958).

The peaks in the death rates that occur with influenza epidemics are well known (Stocks, 1935, Francis, 1953). Stuart-Harris has shown (Stuart-Harris, 1947, Stuart-Harris, Franks and Tyrrell, 1950) that whenever the certified influenza death rates in the Great Towns of England and Wales have been over 100 per week laboratory tests for influenza have been positive. Investigations have shown the connection of pneumonia and influenza (Stuart-Harris, 1947, Stuart-Harris et al., 1950, Finland, Barnes and Samper, 1945) and have supported the contention that the influenza-certified deaths do constitute a valid index of deaths due to this cause. Indeed, there is evidence that deaths certified as due to influenza form only a proportion of the deaths that are in fact caused or precipitated by that disease, because the rise in mortality

so greatly exceeds the expected seasonal rate (Collins, 1957, Roden, 1963). This points out the risk of epidemic influenza to certain groups, particularly - as Short (1749) would have said "such as .... had unsound viscera".

Although Martin (1950) has pointed out that the mortality due to influenza in Britain showed a progressive decline from 1919-1949 it remains a potent cause of death in the community. McDonald (1963) applying a conservative calculation estimates that influenza caused 3% of all deaths in Western Europe from 1955-1959.

However, the age-distribution of deaths is quite unlike that of the attack rate. With one outstanding example the deaths attributed to influenza are massed at the extremities of life. This was noted by observers in centuries past - Arbuthnot (1733, cited by Thompson, 1852) wrote ".... and everywhere the disease was particularly fatal to aged people", and John Huxham of 1743 (1758, cited by Thompson, 1852) ".... sometimes not a little fatal to old men and children who had weak lungs". However, it is much more satisfactorily displayed in the charts of Collins (1944, 1957).

In the description of Asian influenza in a general practice by Fry (1957) the concentration of complications in older age groups is clearly shown.

Although unproven, it is generally accepted that the pandemic of 1918-1919 was caused by an influenza A variant. (Andrewes, 1950, Francis, 1953). In that epidemic, briefly discussed above, the most extraordinary feature was the high mortality in the 25-35 year age group during the autumn of 1918. This resulted in age-specific mortality rates (Collins, 1944) quite unlike those in any other influenza epidemic. In some U.S. military camps a case mortality rate of 3.3% was noted during the autumn of 1918 (Ireland, 1928).

The world-wide death roll of this epidemic has been estimated at between 15 and 20 millions - thus considerably improving upon the ingenuity of man from 1914-1918 - and the epidemic of this normally "mild" disease entered history along with the Black Death and the Plague of Justinian.

Prevention of Influenza:

Thus, the disease once called the "gentle correction" is shown to be a potent cause of death to the old and the young, a plague to the school-child, a financial blow to the industrialist, a burden to the medical officer of health, a general spendthrift of life, time and money and to many in their sixth decade it may be a dreadful memory.

In these circumstances it is right to seek rational

methods of prevention.

1/ Sanitation

Influenza, like many other viral diseases dependent upon the proximity of person to person, has not been much affected by the removal of sewage from that proximity.

Measures of air sterilization (Stokes and Henle, 1942) have not been altogether successful in practice.

2/ Quarantine

The universal nature of the disease makes quarantine normally out of the question, particularly as spread can be caused by the apparently healthy as seen in the Ocean Island incident (Isaacs, Edney, Donnelly and Ingram, 1950).

However, the fact that the spread of influenza is from person to person does mean that total severance of outside connections can protect. This was shown in several sanatoria and institutes and even remote towns in the United States and Britain during 1918 (Jordan, 1927), where freedom from infection continued while vigorous isolation was practised. Australia operated elaborate maritime quarantine from October, 1918, to May, 1919, and escaped the extreme virulence of the epidemics which raged in South Africa and New Zealand during October to December. When influenza occurred in January to June, 1919, it carried a lesser mortality. A similar picture was seen in Israel during the Asian influenza epidemic when the infection did

not cross from the neighbouring Arab countries, cut off by the political situation, but was finally introduced from America later. South Africa maintained a maritime quarantine but the epidemic spread overland after a short delay (Payne, 1961).

Whereas isolation of the necessary degree is impossible for many countries and possible only for a short while for others, in some cases quarantine could delay the introduction of infection and enable other preventive measures to be organised.

3/ Chemotherapy

During the last few years a number of agents active against viruses in vivo have been produced. These also have their disadvantages in that viruses have rapidly become resistant to some (Melnick, Crowther and Barrera-Oro, 1961, Loddo, Schivo and Ferrari, 1963, Smith, 1963) and, presumably, prevention of infection would entail taking regular doses. However, these agents will undoubtedly be developed successfully.

Recently a promising anti-influenzal agent has been announced (Newsweek, April 27th, 1964, p. 35) and while the development of this and similar agents is still at an early stage a new era of influenza prevention may be at hand.

4/ Vaccination

In those virus infections of man where methods of

quarantine and of breaking the chain of transmission have failed, vaccination has been the most effective manner of protecting individuals and communities. The success of poliomyelitis vaccines, in particular, has stimulated interest in this mode of preventing diseases of mankind.

CHAPTER II

INFLUENZA VACCINES

The discovery of the influenzal agent

The development of influenza vaccines had, necessarily, to wait upon the isolation, identification and cultivation of the agent responsible. The "renaissance" of influenza in that late 19th century stimulated enquiry and Pfeiffer (1893) described a small cocco-bacillus, later to be called Haemophilus influenzae, as the cause of influenza. However, as early as 1900 Rosenthal (quoted by Thomson and Thomson, 1933) was of the opinion that Pfeiffer's bacillus was only a secondary invader. During the great pandemic of 1918-19 H. influenzae was only one of the organisms to be isolated from the sputum and lungs of cases and most vaccination attempts using various organisms including H. influenzae yielded highly equivocal results (Ireland, 1928). From their study of the pathology of fatal cases Winternitz (Winternitz, Wason and McNamara, 1920) and Goodpasture and Burnett (1919) stressed the "toxic" effect of the "true etiological agent". The latter authors quoted Leichtensten's (1899, cited by Goodpasture and Burnett, 1919) opinion that there was a primary influenzal pneumonia produced by the poison of influenza. The opinion of these pathologists was that the

bacteria were secondary invaders.

Numerous attempts at isolating infective agents were made during the pandemic. One of the most interesting concerned the isolation by Olitsky and Gates (1921 a & b) of Bacterium pneumosintes.

As late as 1933 Thomson and Thomson (1933) reviewing the immense literature concerning various organisms proposed as the aetiological agent were unable to express any more than vague opinions.

Although the virus of swine influenza in the United States was isolated by Shope (1931) in pigs, it was not until 1933 when Smith, Andrewes and Laidlaw (1933), followed by Francis (1934), isolated influenza in ferrets by intranasal inoculation, that any fundamental knowledge concerning influenza in man could be acquired.

Since that time studies on influenza viruses over three decades have shown that the important agents of influenza can be divided into two fundamentally different types, A and B. Within each type antigenic variations have been shown to occur. These variants can be grouped into "families" on the basis of close relation. These families are more sharply distinguished in Type A virus and have been named A, A1 (previously A-prime) and A2 (Asian).

Immunity to Influenza

It was shown that convalescent ferrets were initially refractory to repeated infection but after some six months could be re-infected with the same strain. However, the disease tended to be milder and, in the case of inoculation under anaesthetic the convalescent animals were protected from lung involvement (Smith et al., 1933, Smith, Andrewes and Laidlaw, 1935). It was also shown that the convalescent ferret possessed a high titre of virus neutralizing antibodies (Smith et al., 1933).

The adaptation of influenza virus to mice by the intranasal route (Andrewes, Laidlaw and Smith, 1934) made possible the development of a practicable neutralization test (Andrewes et al., 1934, Laidlaw, Smith, Andrewes and Dunkin, 1935) for the identification and assay of antibody and of infective virus. Mice could also be passively protected by the administration of neutralizing antibody.

Surveys in human populations showed that neutralizing antibodies (Smith et al., 1933) to influenza virus were widespread (Andrewes, Laidlaw and Smith, 1935) and that there was a definite age distribution in that antibodies were rare in young children, then increased in frequency until the mid-thirties and then declined towards old age. Infants up to six months of age possessed the antibody levels of their mothers (Francis and Magill, 1936).

It was shown that antibodies could be elicited in ferrets, mice, horses (Laidlaw et al., 1935) and rabbits (Francis and Magill, 1935 b) by parenteral inoculations with virus containing material either derived from infected mouse-lung or from a Maitland type tissue culture (Francis and Magill, 1935 a) without the animals developing clinical disease. It was also possible to use virus inactivated by formalin but not by heat or by methylene blue (Smith et al., 1935). Opinions differed about the efficacy of formalinized virus (Smith et al., 1935, Andrewes and Smith, 1937, Francis and Magill, 1936) and this led to rather different approaches to the task of producing antibodies in man.

THE EVOLUTION OF INFLUENZA VACCINES

American workers felt that only a vaccine composed of active virus would suffice and while Francis and Magill (1936) used the super natant fluid of an infected Maitland type of tissue culture as a source of virus - believing that this would be less likely to contain contaminants - Chenoweth, Waltz, Stokes and Gladen (1936) employed a filtrate of infected mouse lung. Francis and Magill inoculated 23 adult volunteers with multiple doses of their preparation. In Chenoweth's experiment a large number of children at a state institution were the subjects.

However, the British workers (Stuart-Harris, Andrewes and Smith, 1938), mindful of Shope's experience when live

swine influenza vaccine had spread from vaccinated swine and caused clinical disease in the rest of the herd (Shope, 1936), used a formalized vaccine prepared from mouse lungs. Special precautions were taken to avoid contamination with the virus of lymphocytic choriomeningitis.

The step of adapting influenza virus to grow in embryonated eggs was inspired by the success of Goodpasture and Woodruff (Goodpasture, Woodruff and Buddingh, 1931, Woodruff and Goodpasture, 1931) with fowl-pox. Burnet was able to grow influenza strains first on the chorio-allantoic membrane (Burnet, 1936) then in the amniotic cavity (Burnet, 1940 a and b, 1941 a). This was to provide a much more simple method of isolating virus (Hirst, 1942 c, Burnet and Stone, 1945). Apparently, the work of Scott (1938) with swine influenza virus had been ignored because it was only in 1941, when Nigg (Nigg, Wilson and Crowley, 1941) and Henle and Chambers (1941) were followed by Burnet (1941 b), that the potentialities of using the high titres of virus obtained in the allantoic fluid for vaccine production were appreciated.

The employment of chick embryos for growing the virus led to the discovery of its haemagglutinating properties (Hirst, 1941, 1942 a and b, McClelland and Hare, 1941) which provided a convenient method for the assay of virus and of antibody.

At this stage a curious development in influenza vaccines took place. Horsfall and his associates accidentally noticed that influenza virus grew to higher titres in the tissues of a ferret simultaneously infected with a certain strain of canine distemper (Horsfall and Lennette, 1940). The observation formed the basis for the production of a "complex vaccine" consisting of formalized allantoic fluid from embryos infected simultaneously with influenza and the specific distemper strain. Trials were carried out on animals and on progressively larger numbers of humans. Although the initial reports on this unusual mixture were favourable (Horsfall, Lennette and Rickard, 1941, Horsfall, Lennette, Rickard and Hirst, 1941) later experience (Martin and Eaton, 1941, Hirst, Rickard, Whitman and Horsfall, 1942) did not encourage further development of this idea.

Concentrated Vaccines:

It had previously been shown that, within limits, the antigenic efficacy of an influenza vaccine was directly related to the amount of virus in the dose (Francis, 1939, Hirst et al., 1942). Therefore the use of methods of concentrating virus to achieve better responses was indicated. As knowledge of the properties of influenza virus increased so did the possible methods of concentration and purification.

One of the first to be attempted used the fact that show, cold thawing of frozen infected allantoic fluids released a precipitate containing the greater part of the virus (Hare, McClelland and Morgan, 1942, Hirst, Rickard and Whitman, 1942). This was developed sufficiently to produce vaccines on a small scale (Hare, Morgan, Jackson and Stamatis, 1943) but on a larger scale difficulties with bacterial contamination limited its use (Francis, 1945).

Francis and Salk (1942) showed that the haemagglutination reaction mentioned above could be adapted for concentration by utilizing the property of the virus, previously absorbed on erythrocytes, to elute after incubation into a smaller volume if required. This red cell elution method was used for the preparation of vaccines on a large scale for the U.S. Armed Forces Epidemiological Board in the trials from 1942-1944 and was only gradually supplanted.

Other methods for the precipitation or absorption of virus that have been described and used to a greater or lesser extent include protamine (Chambers and Henle, 1941), calcium sulphate (Salk, 1941, 1945, Stanley, 1945a), alum (Bodily, Corey and Eaton, 1943) calcium sulphate (Tovarnizky and Ghalkina, 1944), alcohol (Cox, van der Scheer, Aiston and Bohnel, 1947), aluminium phosphate (Himmelweit, 1960) and barium sulphate (Drescher, Hennessy and Davenport,

1962, Mizutani, 1963).

At an early stage it was shown that high speed centrifugation brought down the influenza virus particle associated with infectivity and haemagglutination (Elford and Andrewes, 1936, Henle and Wiener, 1944). Adaptation of the Sharples centrifuge to handle infected allantoic fluid yielded a method capable of producing concentrated and purified virus on a large scale (Stanley, 1944, 1945 and Taylor, Sharp, McLean, Beard and Beard, 1945).

The use of these techniques combined with the knowledge of how to achieve optimal titres of virus in eggs (Beveridge, 1944 a, McLean, Beard, Taylor, Sharp, Beard, Felter and Dingle, 1944, Miller, 1944, Sigurdsson, 1944) underlie the modern success in producing vaccines on a commercial scale.

Adjuvant Vaccines:

The use of simple, inactivated influenza virus in a saline or aqueous base is subject to distinct limitations. Some of these are the toxicity of the virus particles, the cost of producing antigens and the relatively transient nature of the serum antibody elevation. Besides, in time of emergency the amount of antigen that can be prepared is small and the need may be large. Therefore, it was logical to try and explore methods of potentiating the action of the antigen.

By far the most promising method involved the use of mineral-oil emulsions. Freund (Freund and McDermott, 1942, Freund and Bonato, 1944, Freund and Walter, 1944) indicated that by forming a water-in-oil emulsion with mineral oils, killed Mycobacteria and an emulsifying agent and the antigen in the aqueous phase the antibody response could be enormously enhanced. Friedewald (1944) showed that this applied to influenza antigen as well and Henle and Henle (1945) excluded the Mycobacteria from the adjuvant and found, in human volunteers, that the same applied. Salk improved upon the procedure and the ingredients and applied the principle for the immunization of animals and then man (Salk, Lewis, Youngner and Bennett, 1951, Salk and Laurent, 1952).

Oily adjuvant vaccines have now been officially licensed in the United Kingdom for general use.

THE EFFICACY OF INFLUENZA VIRUS VACCINES

The purpose of a vaccine is to protect the recipient against the disease. In this, the various parameters to be considered are the protection ratio, the duration of protection and the lag between administration of the vaccine and the onset of protection. These are all most convincingly tested when the vaccinated population, together with suitable controls, is exposed to the natural

disease. However, with influenza, the epidemic nature of the disease, the difficulty of certain diagnosis, the variation in attack rate and the phenomenon of antigenic variation all tend to aggravate the difficulties of arranging a well-controlled trial and making the success of it unduly dependent upon chance.

Therefore vaccines are frequently most conveniently judged by their antigenicity or their ability to elicit an increase in specific antibody titre which can be readily measured. The relation between serum antibody titre and immunity varies from one disease to another. In the case of influenza this is clearly not absolute. The somewhat heroic experiments of Smorodintseff, Tushinsky, Drobyshevskaya, Korovin and Osetroff (1937) indicated that while antibody titre was an indication of resistance the possession of antibody by no means signified immunity. Evidence from the 1918-1919 pandemic (Ireland, 1928, Jordan, 1927) and from later community studies (Pickles, Burnet and McArthur, 1947, Jordan, Badger and Dingle (1958) show some degree of immunity to be conveyed by natural infection but that this seems to be transient. So much information about the relation between antibody titre and immunity has been obtained from vaccine trials that the efficacy of vaccines will be described from both aspects together and the subject discussed later.

Early Vaccines

The subjects in the pioneering trial of Francis and Magill (1935-6) had been selected for their low level of neutralizing antibody and responded to multiple inoculations of active virus derived from tissue culture supernatant by an increase in antibody titre. In a further similar trial (Francis and Magill, 1937) pre-inoculation sera were compared with samples taken at intervals after the last inoculation. Here the level of antibody was significantly raised at the time the first sample was taken, remained constant for two months and then declined.

Meanwhile, Chenoweth, Stokes and associates (Chenoweth et al., 1936, Stokes, Chenoweth, Waltz, Gladen and Shaw, 1937, and Stokes, McGuiness, Langner and Shaw, 1937) had been conducting trials in an institution with groups of controls and others inoculated with three doses of either A/PR-8 or A/Swine virus. Only with the former virus was a rise in antibody detected and this in only 31% of those vaccinated. However, an outbreak of respiratory disease involved 25% of the institution during the winter and, considering febrile cases, attack rates were:-

Controls	(550)	12.5%
A/Swine vaccine	(138)	12.4%
PR-8 "	(110)	2.7%

Subsequent serological tests showed the outbreak to have been caused by influenza.

In Britain, Stuart-Harris et al. (1938), using their formalized mouse lung vaccine, found that the antibody response was of the same order as that with natural infection and that a second inoculation did not lead to higher titres. However, their attempt to gauge the protection afforded with a trial in 1936-1937 yielded quite inconclusive results partly because the influenza occurred before vaccination was complete and partly because of antigenic variation. It was clear that absolute protection was lacking. Using the same vaccine in Hungary, Taylor and Dreguss (1940) produced a similar result. Two years after the first attempt a second trial also failed to show protective value (Stuart-Harris, Smith and Andrewes, 1940).

Siegel and others (Siegel and Muckenfuss, 1940, 1941, Siegel, Muckenfuss, Schaeffer, Wilcox and Lieder, 1942 a) embarked upon a long, complex trial in a large institution. Initially, two influenza vaccines were used and some of the vaccinated group were re-vaccinated annually. Appropriate serum samples were taken so as to establish the antibody response to the vaccines and to natural infections over a protracted period. Both of the vaccines, despite marked individual variations of the recipients, produced a three-fold increase in antibody but complement-fixing (C.F.) titres returned to the initial level in 2 months. This

pattern was repeated in following years. Two influenza epidemics occurred during the period of study. One of these took place after the subjects had been vaccinated. At this time the antibody levels of the community had declined from their post-vaccination peak and the effect of the epidemic was to return antibody levels to that peak before they started to decline again. The vaccines showed no protective effect. In a continuation of their programme this group tested the "complex vaccine" of Horsfall (Siegel et al., 1942 b). In agreement with Dalldorf, Whitney and Ruskin (1941), they found no protection indicated by their results. As previously mentioned the experiments of Hirst (Hirst et al., 1942 b) showed that the addition of distemper virus had no effect.

Concentrated Influenza Vaccines

Concentrated formalin-inactivated vaccine prepared by the precipitation of virus from frozen allantoic fluid was tested by Hirst (Hirst et al., 1942 a) and by Hare and his associates (Hare et al., 1943). Both groups showed the superiority of the concentrated vaccine in eliciting antibody and the latter confirmed the results of Stuart-Harris et al. (1938) by showing that no better response was achieved by multiple doses of vaccine. A trial to test the protection afforded by this vaccine by Hare, Stamatis and Jackson (1943) was nullified in the absence of a clear-cut influenza epidemic.

Thus, by the early 1940's, it had been established that vaccines of various types caused a rise in serum antibodies whether measured by neutralization tests in mice (Francis and Magill, 1937, Stokes et al., 1937) on the chorio-allantoic membrane of chick embryos (Burnet, Keogh and Lush, 1937), in the amniotic cavity (Burnet, 1941 a), or allantoic cavity (Hirst, 1942). Haemagglutination - inhibition (H.I.) tests were also satisfactory (Bodily and Eaton, 1942) but there was some evidence that C.F. tests were less satisfactory (Eaton and Rickard, 1941). The antibody response to vaccination was similar to that resulting from natural infection (Rickard, Lennette and Horsfall, 1940, Horsfall and Rickard, 1941).

On several occasions it had been noticed that the antibody response to vaccination was greatest in those with the lowest initial antibody level (Eaton and Martin, 1942, Horsfall et al., 1941 a and b) and the latter author found the diagnosis of influenza in those with high initial titres difficult because of the lack of further antibody response.

It had also been shown that cases of influenza tended to occur in those with low titres of antibody (Burnet and Foley, 1940, Rickard, Horsfall, Hirst and Lennette, 1941), but antibody level alone did not appear to be an absolute factor (Stuart-Harris et al., 1938, Hoyle and Fairbrother,

1937, Horsfall et al., 1941b) and therefore, the concept of a critical level (Francis, Magill, Rickard and Beck, 1937) was not wholly acceptable.

Challenge Experiments

In order to test the protective value of vaccines under controlled conditions a number of experiments were devised in which vaccinated individuals inhaled live influenza virus and their responses to this were compared with unvaccinated controls.

Stokes and the Henle's (Stokes and Henle, 1942, Henle, Henle and Stokes, 1943) conducted experiments on children vaccinated by various schedules and challenged either 2 or 4 months later with an inhalation of about 1.8 million mouse L D $_{50}$. 10 of 28 control and 1 of 44 vaccinated children had clinical influenza, and haemagglutination-inhibition (H.I.) tests showed 10 of the 11 cases occurred in the children who had antibody titres of less than 1:256 before challenge.

Francis, Salk and associates carried out rather similar trials on adults. Difficulty was encountered in standardizing the challenge dose. An experiment where unvaccinated individuals were challenged 4 months after an initial exposure showed rather little residual immunity. After vaccination with concentrated virus vaccines the experiments seemed to show that immunity to influenza B was intact 4½ months after vaccination but immunity to influenza A was

waning. All groups that had been vaccinated showed rather less severe disease but because the febrile response was universal the observations were rather difficult to interpret (Francis, Salk, Pearson and Brown, 1944, 1945, Salk, Pearson, Brown and Francis, 1944 b, 1945 b).

Major Vaccine Trials in United States Forces

In the light of the experience accumulated the United States Armed Forces Epidemiological Board embarked upon major trials of influenza vaccine. The absence of an influenza epidemic in 1942-1943 made the first trials futile. However, the following year, using the resources created by war-time mobilization, many thousands of servicemen at universities in different parts of the United States participated in a controlled trial of an inactivated vaccine containing A/PR 8, A/Weiss (Salk, Menke and Francis, 1944 a) and B/Lee (Francis, 1940 b) strains. The vaccine was prepared from infected allantoic fluid and concentrated by red-cell absorption and elution (Commission on Influenza 1944, Francis, 1945).

Epidemic influenza A, preceded by herald cases the previous spring (Salk et al., 1944 a), was widespread in the United States from November, 1943 to January, 1944. Although the clinical disease was mild, the attack rate, particularly in the younger and older age groups, was high (Collins, 1944) and, thus, ideal conditions existed for the vaccine trial.

The protective value of the vaccine was clearly demonstrated at most of the localities and by most groups of workers (Hale and McKee, 1945 b, Hirst, Plummer and Friedewald, 1945, Magill, Plummer, Smillie and Sugg, 1945, Rickard, Thigpen and Crowley, 1945, Salk, Menke and Francis, 1945 b). Overall, the attack rate was 7.11% in the controls and 2.22% in those vaccinated. More than 11,000 men took part in the trial (Commission on Influenza, 1944).

At two centres the epidemics started during the course of vaccination and it appeared that protection was afforded in about a week after inoculation (Hale and McKee, 1945 b, Hirst et al., 1945). Antibody was shown to persist at high levels for at least three months (Salk et al., 1945 a, Magill et al., 1945). Hirst's group argued that immunity waned eight weeks after vaccination as the difference in attack rate between the control and vaccinated group diminished. However, Salk pointed out that this could be due to sub-clinical infection and the exhaustion of the susceptibles among the controls. In California, however, no protection by the vaccine was demonstrated. This result, it was suggested, could be attributed to either the lapse of six weeks between vaccination and the epidemic or to the fact that strains of virus isolated during the epidemic were clearly distinguishable from PR-8 or Weiss strains antigenically (Eaton and Meiklejohn, 1945).

The relation of antibody to susceptibility during these studies was examined by Salk and by Hirst. In Salk's group of cases 82% of clinical disease occurred in the 48% of the population with an antibody titre of 1 in 64 or less. It appeared that antibody titres produced by the vaccine were similar to those induced by infection. However, Hirst noted the occurrence of 38 cases who had mean acute phase titres of 1 in 558.

In addition, the 1943-1944 epidemic afforded some evidence on the duration of the vaccine's effect because groups inoculated for the previous winter were followed up. The vaccine then lacked the Weiss strain isolated in 1943. Hirst (Hirst, Rickard and Friedewald, 1944) studied a prison population and found that H.I. antibodies to PR-8 rose by 2 weeks after inoculation to 6 times the initial titre. In 11-14 months the levels were at 3 times the original titre. However, the protection ratio was only 35%.

Salk (Salk, Pearson, Brown, Smyth and Francis, 1945 c) demonstrated the maintenance of vaccine-derived antibody by showing that the percentage of subjects with titres of 1:256 or more varied as follows:-

Initial	2 weeks	4 months	12 months
15%	83%	69%	66%

During the course of the year the composition of his test population altered to make a reliable estimate of

attack rates difficult.

The data was as follows (Salk and Francis, 1946):-

	No. wards	Total population	Attack Rates Overall	Maximum	
Unvaccinated	15	1319	12.4%	29.1%	(In 8/15 wards, Rate 10%)
Partly vaccinated	20	1916	1.9%	6.5%	(In 16/20 wards, Rate 4.4%)

The authors felt that this suggested that the presence of individuals vaccinated a year previously had reduced the intensity of the epidemic and thus afforded a measure of protection to the whole community, vaccinated and unvaccinated alike.

It is apparent that differences of opinion occurred. From the available evidence Hirst felt that it was essential to vaccinate in the face of an epidemic because of the rapidly waning immunity. Salk appeared to believe that immunity declined more gradually, parallel with the antibody titre.

In brief, this first major trial showed that influenza vaccine could produce very substantial but by no means absolute protection with a single inoculation. It confirmed that the titre of circulating antibody and immunity were, in the main, related. The length of time

needed for vaccination to take effect was demonstrated but no definite answer was provided concerning the duration of the immunity conferred.

Influenza B, 1945-46.

The end of the world war in 1945 was accompanied by a fear that the chaos prevalent might somehow tend to favour the occurrence of a lethal influenza pandemic. It was noted that the lapse of time between 1890 to 1918 was not dissimilar to that from 1918 to 1945 and an intensive influenza surveillance scheme was commenced in Britain and elsewhere.

However, the epidemic that arrived was of influenza B, spreading from the East and causing epidemics in the Pacific, Australia, Alaska and the Caribbean (Burnet, Stone and Anderson, 1946, Jackson, 1946) between March and November, 1945. Sharp epidemics occurred in the United States in November and December and in Britain from December, 1945, to March, 1946. Although strains of virus isolated were only partly related to the Lee strain used British and American workers were able to indicate that the standard vaccine had provided substantial protection.

Hirst, Vilchers, Rogers and Robbins (1947) compared the attack rates occurring in two groups at Yale University. Francis (Francis, Salk and Brace, 1946) studied groups at university in Michigan.

	Attack Rates	
	Controls	Vaccinated
Yale	12.5%	0.5%
Michigan	9.9%	1.15%

Dudgeon, in Britain (Dudgeon, Stuart-Harris, Andrewes, Glover and Bradley, 1946) was able to compare attack rates in Glasgow and at Woolwich although vaccination was not started until the epidemic had begun.

	Attack Rates	
	Controls	Vaccinated
Glasgow	8.6%	1.7%
Woolwich	10.9%	5.1%

Influenza A, 1946-1947.

Studies of the periodicity of influenza (Commission on Acute Respiratory Diseases, 1946) had led to the expectation of influenza A in 1946-7. As a result trials involving tens of thousands of subjects on both sides of the Atlantic served to illuminate the limitations of the current vaccine the more sharply.

The epidemic spread from the East once more with outbreaks in Australia, Japan and Korea in 1946. The United States was invaded in January, 1947, and many military establishments had outbreaks during the next two months. There was no evidence that the current influenza vaccine

had been effective (Sartwell and Long, 1948). When the civilian population was affected, further reports of the failure of the vaccines occurred (Fowle and Weightman, 1947, Francis, Salk and Quilligan, 1947, Loosli, Schoenberger and Barnett, 1948, Sigel, Schaffer, Kirber, Light, and Henle, 1948).

In the United Kingdom monavelent A virus vaccines containing either PR-8 or Mel (Burnet, 1935) had been used. 20,000 people had been inoculated. The attack rate was so low as to render much of the work useless but outbreaks at schools and army camps showed the vaccine to be virtually ineffective (Mellanby, Dudgeon, Andrewes and MacKay, 1948).

Strains of the epidemic virus isolated in the United States, e.g. FM_1 (Rasmussen, Stokes and Smadel, 1948) and the CAM strain isolated in Australia in 1946 were found to be similar and these were markedly different from the older strains PR-8, Weiss, etc. Although infection with the new strains was shown to cause an anamnestic rise in antibodies to the PR-8 strain (Stuart-Harris, Laird, Tyrrell, Kelsall, Franks and Pownall, 1949) the degree of antigenic relation was insufficient for the older vaccines either to elicit antibody to, or protect mice from, the new variants.

Although during this epidemic the attack rates were relatively low and the clinical disease generally mild the

degree of antigenic variation that had occurred was a highly significant discovery. Previously, although minor antigenic variation had been shown to take place both in Type A (Magill and Francis, 1936, Burnet, 1937, Magill and Francis, 1938, Francis and Magill, 1938, Stuart-Harris et al., 1938, Taylor and Dreguss, 1940) and Type B (Eaton and Beck, 1941, Gordon, 1942) the overall success of the trials in 1943 and 1945 had led to this being discounted as a factor in vaccine production. It had been thought that it was only necessary to select a highly antigenic and "intermediate" strain of each type (Smith and Andrewes, 1938).

Vaccines against A_1 Strains

When the new strains were incorporated into vaccines it was noticed that they were not necessarily as efficient antigenically as the older PR-8 (Salk, Laurent and McGinnis, 1949) and that vaccination against a completely new variant presented difficulties (Meiklejohn and Bruyn, 1949, Appelby, Himmelweit and Stuart-Harris, 1951). However, a series of trials were prepared to examine the efficacy of the new vaccines.

In 1947-8 the work of Salk and Suriano (1949) seemed to show, in rather unfavourable conditions, that, unlike the old vaccine, a vaccine containing A/FM_1 gave protection.

Using monovalent vaccines containing PR-8 or FM_1 Meiklejohn, Weiss, Shragg and Lennette (1952 b) further studied the relation between the old and new families of A virus. The following winter the same group showed that an FM_1 vaccine conferred protection in contrast to a PR-8 vaccine and to controls. The tendency for clinical cases to occur in those with low titres of H.I. antibody was confirmed (Meiklejohn, Kempe, Thalman and Lennette, 1952 b).

At this time the Medical Research Council, in Britain, set up a Medical Research Council Committee on Clinical Trials of Influenza Vaccine (M.R.C. 1953) to study the use of influenza vaccines. A series of field trials were organised in which varying preparations of vaccine were investigated. An outstanding feature of these trials was that large numbers of volunteers were used in many parts of the country and from varied walks of life. Attack rates tended to be low but it was shown that vaccines containing A_1 strains conferred protection against the homologous virus (M.R.C. 1953, 1957). Hawkins, Hatch and McDonald (1956), however, showed much more dramatic results in a school epidemic. Attack rates were as follows:-

Groups	Numbers	Attack rates
Uninoculated	344	20%
Vaccinated in 1954	120	12%
" " 1955	100	8%
" " 1954 and 1955	100	2%

It appeared here that some degree of protection was provided by vaccination a year previously.

Asian Influenza

Therefore, when reports from Singapore, Japan and Taiwan in 1957 gave warning of epidemic influenza A virus of a grossly different antigenic character (Mayer, Hilleman, Miesse, Crawford and Bankhead, 1957, Jensen, 1957 a) much knowledge concerning vaccine manufacture was available and great efforts were made to produce suitable vaccine.

The rapidity of spread, combined with the delayed reporting of the new variant (Payne, 1961) made it very difficult to arrange large scale trials. However, an experiment in which vaccinated volunteers in the United States were challenged with live virus from human sources showed that protection or modification of the disease occurred (Bell, Ward, Kapikian, Shelokov, Reichelderfer and Huebner, 1957).

It was possible as a result of chance or deliberate trial to examine the effect of vaccines containing older A

strains (A/Swine, A, and A 1) and compare this with the result of the somewhat hastily prepared Asian (A_2) type vaccines. Some differences of opinion occurred. Whereas A_2 vaccines showed a protection effect in all cases Gundelfinger, Stille and Bell (1958) felt that the older type also exerted some slight effect. However, in a troopship outbreak (Schreiber, 1957) no benefit was conferred by the older vaccine and British studies in schools (M.R.C. 1958) confirmed this. Like Gundelfinger et al (1948) Culver (Culver, Nitz and Lennette, 1957) showed that monovalent A 2 type vaccine became effective 10 days after inoculation.

Culver also demonstrated, in the same trial, that as with the early A1 vaccines, serological response to vaccination was poor. In an investigation after the autumn epidemic of Asian influenza it was shown (Holland, Isaacs, Clarke and Heath, 1958) that the antibody response of volunteers to vaccine was conditioned by their previous experience with the antigen. People who had no pre-inoculation antibody responded poorly to the first inoculation. Those who had antibody before the first injection produced higher titres of antibody but did not show a further response to a second inoculation. Despite this poor antibody response to a single inoculation protection was shown to be conferred by this (M.R.C. 1958).

In addition to the lack of time the poor titres of virus that were initially found in the allantoic cavity of infected eggs resulted in a shortage of vaccine. As a result attempts were made to "stretch" the vaccine by using smaller doses intradermally Love, 1957, Sigel, Edwards, Schlaepfer, Wellings and Beasley, 1957, Sanger, 1959). This had been originally suggested by Van Gelder, Greenspan and Dufresne (1947, cited by Sanger, 1959) and discounted (Bruyn, Meiklejohn and Brainerd, 1949, Appelby et al., 1951). While at this time of need the technique of intradermal inoculation regained adherents and rather confusing results were published Boger and Liu (1957) showed that 0.1 ml. of vaccine administered intradermally gave a rather poor serological response. McCarroll and Kilbourne (1958) studied this problem carefully and showed that intradermal inoculation possessed no distinct advantage over the subcutaneous route and that the antibody response was related to the mass of antigen administered. However, in the case of the new antigen more than one inoculation was needed to produce an adequate antibody response.

Adjuvant Vaccines

Experiments with influenza vaccines made up in the form of water-in-oil emulsions have been in progress for the last 20 years. From the first animal experiments by

Friedewald (1944) it has been repeatedly demonstrated that vaccines in this form have achieved much higher titres than those in a conventional aqueous base (Henle and Henle, 1945, Salk, Bailey and Laurent, 1952, Himmelweit, 1960), that a much smaller antigenic mass will achieve a satisfactory response (Salk, Contakos, Laurent, Sorenson, Rapalsk, Simmons and Sandberg, 1953, Davenport, Hennessy and Bell, 1962) and that antibody levels are better maintained (Salk et al., 1953, M.R.C., 1957, Davenport et al., 1962). Whereas Salk (Salk et al., 1953) felt that with vaccines "adjuvanted" thus, the antibody response was "broader", Davenport (Davenport and Hennessy, 1957, Davenport et al., 1962) showed that this was only true within a "family" of viruses.

However, there is not a great deal of evidence concerning the protective value of these vaccines. Philip et al., (1957) seemed to show that definite protection against influenza B was afforded 13 weeks after an adjuvant vaccine, but against influenza A, 60 weeks after vaccination, the value of the vaccine, while present, was less evident. Davenport's trial in 1954-1955 (Davenport, Hennessy, Houser and Cryns, 1956 b) was complicated by the emergence of a new family of Type B viruses (Woolridge, DeMeio, Whiteside and Seal, 1955) and because so many cases occurred less than a week after vaccination. The following year a trial

in Britain (M.R.C., 1957) seemed to indicate that the adjuvant vaccine gave no protection against influenza a year later although the attack rates were very low and the mode of diagnosis would tend to weigh the results against the vaccine (McDonald and Andrews, 1955).

At present there does not appear to be clear evidence that the adjuvant vaccines can give protection for as long as the raised serum antibody titres would seem to indicate.

The Components of Vaccines

It is eloquent of the problems raised by the variable nature of influenza virus that there exist fundamental disagreements on the basic concepts of making up influenza vaccines.

In Britain the antigenic ingredients of vaccines are governed by the feeling that vaccines must attempt, if with imperfect success, to keep pace with the antigenic variations of the influenza virus. It is pointed out that Type A viruses and, to a less marked extent, Type B viruses, can be classified in "sets" or "families". (Jensen, 1957 b). When each new "family" arises it dominates the influenza scene for 10 to 15 years and is itself replaced. Greater or smaller degrees of variation in antigenic and other characters occur within the family. Vaccines, and the antibody produced, specific for one family will afford little or no protection against the next family and that some of the "intra-familiar" variations also may, to a less extent,

render vaccines out-of-date.

In the light of this belief, and the feeling that extraneous antigens merely divert the antibody forming mechanism and may reduce the specific response (British Medical Journal, 1961), it is felt that vaccines should contain representatives of those types of strains which are most likely to cause influenza in the coming season. It is hoped that from the surveillance centring on the World Influenza Centre (Andrewes, 1953) an attempt should be made to predict these.

In the United States of America a different view, that of the "Ann Arbor" school, exists, which has been proposed by Francis (1953, 1954, 1959), Davenport and Hennessey (Davenport and Hennessey, 1960) and is responsible for the concept governing vaccine manufacture in that country. This opinion holds that the British School is pessimistic as it can only always be behind the influenza virus (Davenport, 1958). On the contrary, it is felt that the available evidence points the way to a schedule of vaccination that would protect against influenza of all types.

It is felt that experiments with absorbed sera show the presence of numerous minor antigens which cross-react with strains of widely differing chronological eras and that although certain dominant antigens may characterize a "family" those antigens will still be present in other

families (Jensen and Francis, 1953, Jensen, 1957 b). This leads to the concept that the range of antigenic variation of either of the two types, A or B, is limited. Therefore, epidemic influenza is felt to have a cyclical basis (the Einsteinian or "Franciscan" curve of Andrewes, 1957) so that, in response to selection pressures different groups of antigens dominate in turn. From this it should be possible to incorporate all the important antigens that can characterize influenza to form a totipotential vaccine.

The lower incidence of influenza among older people is held to be due to the nature of their antibodies to influenza viruses. The antibody heritage of the first infection in life will dominate the antibody picture thereafter so that a chronological map of influenza can be drawn up showing when "sets" of strains were prevalent. This is the "doctrine of original antigenic sin" (Davenport, 1958). However, repeated infection with different strains leads to a broad-based composite antibody which is responsible for the relative insusceptibility of older people (Davenport, Hennessy and Francis, 1956, Hennessey and Davenport, 1961). The "Ann Arbor" school feels that vaccine policy should be directed at creating a similar type of antibody in younger people by incorporating multiple strains of each type. In this context it is believed that the trial reported by Gundelfinger et al. (1958), which appeared to show that a

vaccine containing A and A_1 antigens only gave partial protection against an A_2 epidemic, indicated that the minor, shared, antigens were important and lent support for the concept of a polyvalent vaccine.

Mulder and Masurel (1958) reported that old people in Holland possessed antibody to the A_2 influenza virus before the epidemic. Davenport (1958) confirmed this finding in America and both groups suggested, on this basis, that the epidemic of 1889-1890 had been caused by an Asian type of virus and therefore the full range of major antigenic variations within the capacity of type A influenza virus have now been displayed.

REACTIONS

Although the more lurid pictures of the anti-cowpox vaccination cartoonists, depicting people turning into cows, did not turn out to be justified, it is true that the use of any vaccine may be limited by undesirable reactions and side effects.

These reactions may clearly be caused by the mode of administration (e.g., inoculation), the vehicle (e.g., aqueous, alum absorbed, or adjuvant), extraneous impurities, the toxicity of the antigen itself, or, in the case of living vaccines, may result from the normal or abnormal multiplication of the vaccinating agent or contamination with undetected organisms.

Early Vaccines

From the earliest trials critical observers noted reactions to the administration of vaccines. Francis and Magill (1935-1936, 1937) using a preparation of live virus grown in tissue culture recorded mild erythema and sometimes slight tenderness over the site of the inoculation. No pyrexia was noted in their volunteers. Stokes et al. (1937 b) found that with a vaccine of active mouse lung a mild local reaction occurred in a few of their 250 subjects. One person had a marked reaction locally. When a tissue culture vaccine was used these workers noted no local reactions but a few of their numerous subjects had common-cold-like symptoms after inoculation. Stokes' trials were carried out on feeble-minded children which might have affected the recording of responses.

With similar subjects and a similar vaccine Siegel and Muckenfuss (1941) found a 15% incidence of local redness and swelling which disappeared within 24 hours. General reactions were rare but several children complained of headache and head colds 24-48 hours after inoculation.

Reactions were not recorded in the earliest British trials (Stuart-Harris et al., 1938) where a formalized mouse lung vaccine was used. However, with a similar vaccine in 1939 (Stuart-Harris et al., 1940) only mild local reactions were observed.

By the 1940's vaccines based upon infected allantoic fluids became available and Brown (Brown, Eaton, Meiklejohn, Lagen and Kerr, 1941) recorded that following the use of a formolized "complex" distemper-influenza vaccine, local tenderness for 3-4 days was common.

Concentrated Vaccines

With the introduction of formolized vaccines concentrated by various means (and, possibly, also more carefully studied trials) the reactions observed increased in frequency and severity. While Hare's subjects (Hare et al., 1943) noticed a considerable amount of stinging - probably from excess formaldehyde - local reactions were common but general reactions were very few. Hirst et al. (1944) found that 1 in 200 of those receiving 1 ml. of vaccine developed a fever of 100-100.5° F. and a moderately sore arm with redness and swelling for 24 hours at the site of inoculation was common. With vaccine prepared the same year Salk et al. (1945 c) reported that systemic reactions occurred in 2% (3 persons having a temperature of 101° F.) and local reactions occurred in 65% of 600 subjects after vaccination. No reactions occurred in controls who were inoculated with a placebo.

During the large scale trial of 1943-1944 different groups paid differing degrees of attention to reactions. Those in the trial received either 1 ml. of vaccine subcutaneously or a similar volume of saline containing

formalin at the same strength as the vaccine. Salk (Salk et al., 1945 a) noted that while the control inoculation produced virtually no ill effects 1.46% of the vaccinated group reported sick and of 50 of these, 9 had a fever of 100° F. or more. On questioning, 48% reported a systemic reaction and 73% local reactions consisting of tender areas with erythema and induration.

Eaton and Meiklejohn (1945) interviewed samples of 116 men from each group and classified reactions as mild and moderate.

	Systemic		Local	
	Mild	Moderate	Mild	Moderate
Vaccine Group	6	21	18	18
Control Group	0	2	8	3

Of the whole group of 1,761 men, 40 reported to the dispensary as a result of inoculation.

Rickard et al., (1945) inoculated 1,206 men and circulated questionnaires to both vaccine and control groups 48 hours after vaccination.

	Number	Replies	No Reaction	Local Reaction	Systemic Reaction
Controls	607	539	90%	10%	0.7%
Vaccine	599	550	9%	91%	18%

Four men receiving vaccine were sufficiently ill to be admitted to hospital for 12-24 hours.

In the Iowa University study of 599 receiving vaccine 39 had general symptoms and 32 had sufficiently severe local reactions to limit the use of an arm for a day (Hale and McKee, 1945 a).

It is clear that the number of reactions was significant. In addition it was clearly demonstrated in the controls that neither the injection nor the formalin was responsible for the symptoms.

In an industrial group with a more mixed population Norwood and Sachs (1947) found that whereas reactions sufficiently severe to cause loss of time from work were rare a formidable number of complaints appeared in answer to their questionnaire.

Local Reactions

Sore arm	77.3%

General Reactions

Elevated temperature	29.5%
Headache	37%
Aching muscles	40%
Nausea and vomiting	6.5%

It is not surprising that Salk and Francis (1946) equated the reactions to influenza vaccine with those caused by typhoid vaccine. However, in a trial in 1947 Francis (Francis et al., 1947) inoculated 10,000 students and less than 1% had systemic complaints. Although the

vaccine was of similar haemagglutinating potency (5,120 H.A.U.) to that used in 1943 it is perhaps significant that it was due to expire shortly after it was used.

It had been shown on several occasions that influenza virus shows an inherent toxicity (Henle, G. and Henle, W., 1944, 1946, Hale and McKee, 1945 b, Evans and Rickard, 1945, and later, Sugg, 1949). Many of these studies showed that the toxicity was destroyed by formalin. However, the studies of Salk (1947, 1948) established that the febrile, influenza-like symptoms that had been noticed in a proportion of those receiving vaccine were a property of the virus, not of the impurities that might be present. The nature of the reaction was not settled but the evidence seemed to suggest that a "toxic" effect was involved rather than sensitization.

In an extensive survey of reactions Sadusk, Bassett and Meddaugh (1949) compared two vaccines, one prepared by red cell elution and one by centrifugation techniques. The vaccines both seem to have contained a very large antigenic mass - 1,400 chicken cell agglutinating units (C.C.A.) (Hirst and Pickels, 1942, Miller and Stanley, 1944). No difference was found between the vaccines but females and the younger age groups of this large office employee population were more affected than males and people of 25 years and older, respectively. In this trial also,

systemic reactions were reported in up to 36%, and local reactions in 60% of those inoculated.

British trials at various times (M.R.C., 1955, 1958, Himmelweit, 1960, Cope, 1960, Norman and Rainsbury, 1960) showed the proportion of reactions occurring with aqueous based vaccines. In 1953 a saline vaccine containing 20,000 H.A.U. caused:-

General Reactions	Local Reactions		
	Erythema	Swelling	Induration
7.6%	40%	45%	32.8%

In four cases the induration persisted for a year.

Norman and Rainsbury (1960) recorded a few complaints of painful arms for two or three days only and in a series of 3,209 studied by Cope (1960) about "half a dozen" were absent from work for 1-2 days after vaccination. Two of his patients reported extensive local reactions.

Himmelweit's series with 400 volunteers, mostly less than 25 years old, showed systemic reactions in 2% and local reactions in 3.6%. These figures are markedly lower than in the American figures but whereas the report of the trial in senior schoolboys by Hawkins et al. (1956) does not contain a note about reactions it appears that these were fairly severe.

Two interesting reviews of reactions in the United States armed forces were published by Seal (1955) and

Griffin (1959). Seal reported on the effects of influenza vaccine at Naval and Marine training centres and showed that the proportion of those hospitalized in 1955 was considerably under 1:1000. However, in one group 80% complained of some symptom referable to the inoculation. More detailed studies on smaller groups of officers and hospital corpsmen confirmed a high incidence of reactions.

Griffin surveyed vaccination in the Army and noted the difficulty of getting reliable accounts, showing that in 1945 the incidence of generalized reactions in different reports varied from 7% - 85%! Therefore, Griffin used time lost from duty as an index and counted the number of attendances at out-patients after inoculation with influenza vaccine from 1953-1955. During this period about 3½ million doses of influenza vaccine were given. Reactions which caused the recipient to attend out-patients occurred in 0.6%. 97% of these reactions were treated while the men remained on duty. In regard to reactions, influenza vaccines were shown to be strictly comparable with booster doses of typhoid or tetanus vaccines and much less prone to cause reactions than small-pox vaccination. An interesting fact is revealed by the steadily decreasing rate of reactions, from 11.83/1000 in 1953 to 2.83/1000 in 1955.

Influenza Vaccine in Children

Although some of the earliest trials were carried out in children these vaccines, by later standards, contained very little antigen. Grant (1946) and Salk (1947) pointed out that infants and young children suffered worse reactions with less virus. Quilligan and colleagues (Quilligan, Minuse and Francis, 1948) had to alter the doses used in an investigation in children because of the severe febrile reactions and confirmed the frequency and severity of them (Quilligan, Francis and Minuse, 1949). In a group of children, average age 3.2 years, given vaccine with 1,024 H.A.U., 74% developed temperatures of over 100° F. Even 128 H.A.U. given as a first inoculation caused fever in nearly 20% of the recipients.

Higgons, Nigg, Hyde and Mann (1948) tried various doses and routes of administration in children aged from 3 months to 12 years. Doses of 0.1 ml. intradermally and 0.2 ml. sub-cutaneously were well tolerated but the antibody response was very poor. 0.5 ml. sub-cutaneously caused febrile reactions in nearly 40% but all those with higher fevers (100.8° F.) were less than 5 years of age. Bruyn, Meiklejohn and Brainerd (1949) made a careful study comparing sub-cutaneous doses graduated for age, and intra-dermal vaccine. With sub-cutaneous vaccine about 50% of all ages had systemic reactions of one sort

or another and local reactions causing limitation of arm movement in a third.

Davenport and Hennessy (1960) recommend low dosage schedules for young children in view of the frequency of reactions.

Adjuvant Vaccines

The early experiments of Henle (Henle and Henle, 1945, Henle, Henle, Hampil, Mauris and Stokes, 1946) showed that with the adjuvant vaccines they used, the great advantages in antibody production were off-set by the high incidence of local reactions with residual nodules and sterile abscesses. However, the finding that administration in oil emulsion reduced the toxicity of other substances (Halbert, Smolens and Mudd, 1945) and the possibility of being able to use a smaller antigenic mass to obtain the same antibody response awakened the promise of reduced systemic reactions.

Salk (Salk et al, 1951, Salk, Bailey and Laurent, 1952, Salk and Laurent, 1952) showed that by using Arlacel A, which Freund had used (Freund, Thomson, Hough, Sommer and Pisani, 1948, Freund, Thomson, Sommer, Walter and Pisani, 1948), as an emulsifying agent and Bayol F as mineral oil and injecting the emulsion intramuscularly, he could avoid the undesirable reactions that were reported previously and retain the antibody response associated with the water-in-oil emulsion.

Further trials in people were commenced both in the United States and Britain using Arlacel A and Bayol F, but in both countries there were reports of chronic reactions (M.R.C., 1955, Philip et al., 1954). In Britain, the two cases were of a minor nature but in the American trial a number of sterile abscesses occurred, particularly in negroes. The much larger British trial of 1954-1955 produced rates of chronic reaction of 0.2% and 0.5% in two groups given adjuvant vaccine (M.R.C., 1957). In 9 volunteers the sterile abscesses that resulted required surgical treatment. A retrospective survey of U.S. Army cases at Fort Dix revealed a total incidence of cystic complications of nearly 3.0% (Beebe, Simon and Vivona, 1964). It was found that early batches of Arlacel A were toxic and in further studies in America a partly purified Arlacel was used and Drakeol 6 VR substituted for Bayol F. This, however, still resulted in an incidence of cysts of 0.1% (Philip et al., 1954, Bell, Philip, Davis, Beem, Beigelman, Engler, Mellin, Johnson and Lerner, 1961). Smaller trials since have not reported this complication (Davenport et al., 1956, Hennessy and Davenport, 1961) and Himmelweit (1960) observed no immediate or delayed reactions in his group of 180 volunteers who were examined a year after inoculation with these reactions in mind. However, Furstenberg (1963), reviewing the use of emulsions for desensitization, reported

that cysts still occur occasionally but no details were supplied.

Allergic Reactions

In view of the fact that a proportion of the population is sensitive to egg protein fears were aroused that allergic reactions might occur with vaccines prepared from allantoic fluid (Knight, 1944). Plotz (1946) surveyed the history of the numerous vaccines prepared in eggs and cited a case of anaphylactoid death after influenza vaccine in a soldier who had a life-long history of egg sensitivity.

Ratner and Untract (1946) tested a group of highly allergic children and found that all the cases who reacted to skin tests with influenza vaccine were hypersensitive to egg white. They felt that of their total group of over a hundred, influenza vaccination would be hazardous in 4.6%. Sadusk et al. (1949) skin-tested 79 of their volunteers who suffered from asthma, hay fever, egg or chicken sensitivity or had had a reaction with influenza vaccine previously. After testing, only 16 were rejected and the reactions in the rest, who were vaccinated, were no worse than in the general group. However, Seal (1955) reported 2 anaphylactic reactions occurring in egg sensitive individuals and Griffin (1959) found 3 such cases in his survey of inoculations in 1945, two of which

were fatal. Warren (1956) reported a case with encephalopathy of probable allergic background and Denny (1948) described a case of severe urticaria in an infant without any previous history of allergy and advised that routine precautions should be taken when influenza vaccine was being given.

Davenport (1962) summarized the situation stating that influenza vaccine should not be administered to people who could not eat eggs because of hypersensitivity.

Side-Effects

In addition to the varied reactions that are directly and readily attributable to influenza vaccines some other possible undesirable effects have been discussed.

A) It has been suggested by Springer and Tritel (1962) that as chick embryo tissue contains group A-substance and as the vaccine material is derived from egg allantoic fluid sufficient A-substance could contaminate the vaccine to immunize recipients who are Group O or B. It was further implied elsewhere that this could lead to ABO haemolytic disease in infants of mothers thus immunized. Evidence of a rise in anti-A isoagglutinins in response to inoculation of a concentrated and dialysed extract of influenza vaccine was presented.

However, it was stated by other authors (Influenza Surveillance, 1962, Mathieson, Banner and Harris, 1963)

that this had not been observed to happen. In addition, Sussman and Pretshold (1963) failed to find Group A substance in the influenza vaccines supplied by two manufacturers.

B) Various long-term side-effects that could be associated with the use of adjuvants have been suggested.

First, the possibility has been raised that the mineral oil used may be carcinogenic particularly as it is insusceptible to destruction or disposal by metabolic action. Salk (1961) pointed out that these oils are straight-chain hydrocarbons without cyclical rings and are free from that fluorescence in ultra-violet light associated with carcinogenic hydrocarbons. Also, similar oils have formed deposits in people using them as laxatives or nose-drops but have not been associated with neoplastic disease. Potter and Boyce (1962) showed that the intra-peritoneal inoculation of adjuvants into BALB/cAnN mice causes plasma cell tumours. However, Lieberman (Lieberman, Mantel and Humphrey, 1961) found that this property is restricted to this rather specialised strain of mice and feels that the carcinogenic action of oily adjuvant may be strain specific for this breed. The follow-up studies of Beebe (Beebe et al., 1964) showed that the incidence of neoplasms in those receiving adjuvant vaccine was less than in the group getting aqueous based vaccine. However, the

authors add the reservation that the study has only lasted ten years.

Second, it was feared that the presence of adjuvant might cause sensitization to extraneous stimuli and possibly be associated with auto-immune disease. The experimental work of Rothbard and Watson (1959) is cited as showing the effect of adjuvant in potentiating the effect of the effect of anticollagen serum. On follow-up there is no suggestion in the samples surveyed by Beebe et al. (1964) of an increased incidence of collagen disease or auto-immune disease. However, there are indications of an excess of allergic phenomena, particularly to penicillin, occurring in those people given vaccine between 1951 and 1953. It is not clear whether this could be associated with minute amounts of penicillin in the vaccine or whether the presence of the adjuvant predisposed the individual to become hypersensitive to material given quite adventitiously. In this connection Freund (Freund and Walter, 1944, Freund, 1957) found that adjuvants without the mixture of Mycobacteria had little effect upon sensitization.

CHAPTER III

EXPERIMENTAL

SECTION A

PLAN AND ORGANISATION OF TRIALS

This study was undertaken for a number of reasons.

1 To examine reactions arising from the use of current commercial influenza vaccines.

The review by Griffin (1959) seemed to indicate that influenza vaccines were improving and causing fewer reactions. However, it has always been difficult to compare American vaccines directly with British ones partly because of the polyvalent nature of the former but also because of the different units used. The C.C.A. unit used in the United States is not exactly comparable with the H.A. unit used in Britain. Differing estimates for the equivalence have been given:-

1 C.C.A. unit = 10 H.A. units (Francis, 1959)

1 C.C.A. unit = 25-40 H.A. units (M.R.C., 1958, Himmelweit, 1963)

In addition, the method of assessing reactions has differed markedly from one worker to another and it is difficult to compare the results. With large numbers of people a questionnaire system is the only one practicable. This has its advantages in eliciting the subjective feelings of those inoculated but also tends to select out

those with complaints and simultaneously suggests reactions that might occur.

It is not easy for workers to be objective in reporting the effects of a prophylactic whose use they have proposed. When the observations of Clarke (1962) and of Meichen (Meichen, Rogan and Howell, 1962), which indicated surprisingly high levels of severe reactions, came to view it was felt that a prospective study was required to assess these directly without the complicating factors present in industrial populations. For instance, a "home match" might have a startling effect upon absences due to vaccine reaction.

2 To examine the antibody response to commercially available influenza vaccine.

In addition to the difficulties introduced by divergences in composition and unitage as mentioned above many trials have been performed with univalent vaccines. As British commercial vaccines contain both influenza A and B material it was felt that it would be fruitful to examine the responses to both components of the vaccine.

3 To observe the effect of influenza vaccines upon the titres of anti-A isoagglutinins.

As the article by Springer and Tritel (1962) appeared while the first trial was in progress the

opportunity was taken to carry out limited investigations on the effect of influenza vaccines on antibodies to A-substance.

Plan of Trials

Two separate trials were run over the winters 1962-1963 and 1963-1964.

Trial 1 (November, 1962-November, 1963)

Programme

Part I

A. Volunteers were bled and inoculated with one of two vaccines at a morning session.

B. Volunteers were examined for reactions

i Some hours after inoculation (i.e. the same afternoon)

ii 1 day after inoculation

iii 2 days after inoculation

iv 3 days after inoculation

v 7 days after inoculation

C. 21 days after inoculation volunteers were bled and examined for reactions.

Part II (2 months after commencement of Part I)

A. The same volunteers as in Part I were bled and inoculated with one of two vaccines in a morning session.

B. Volunteers were examined for reactions as in Part I.

C. 28 days after inoculation volunteers were bled and

examined for reactions.

D. 9-10 months after inoculation volunteers were bled and examined for delayed reactions.

Volunteers

Volunteers were drawn from students of the first to fifth years of the Faculty of Medicine at the Queen's University of Belfast.

Each class was addressed verbally and support for the trial was requested. Explanatory sheets and Consent Forms (Appendix 1) were circulated and the latter were collected by the class representatives when completed. It was explained that people who were sensitive to eggs or who suffered from asthma were to be excluded.

Vaccines

The vaccines were kindly supplied by Glaxo Laboratories Ltd.

Part I

Two vaccines were sent labelled as "W" and "R". The vaccine fluids were indistinguishable to cursory examination. The nature of each was not revealed by the supplier until after the trial was over.

"W" was a placebo, consisting of phosphate-buffered saline.

"R" was a saline-based (aqueous) formalin-inactivated vaccine containing:

A/Singapore/1/57 - 7500 H.A.U.
A/England/1/61 - 2500 H.A.U.
B/England/939/59 - 5000 H.A.U. per millilitre.

This was a commercially marketed product. The viruses were grown in the allantoic cavities of embryonated eggs, inactivated with formalin, concentrated and purified by differential centrifugation and diluted to the required concentration with phosphate-buffered saline. The final product did not contain free formalin but had 0.013% thiomersal added as a preservative.

In Part I of the trial 0.5 ml. of either were given by deep sub-cutaneous inoculation over the triceps.

Part II

Vaccines were supplied by the same firm.

"R" was an aqueous vaccine identical to that in Part I.

"O" was a water-in-oil emulsion vaccine dispensed in single dose cartridges. This adjuvant vaccine contained:

1 Aqueous phase:
A/Singapore/1/57 1500 H.A.U.
A/England/1/61 500 H.A.U.
B/England/939/59 1000 H.A.U. per dose

2 Oil phase:
Drakeol 6 V R 9 parts
Arlacel A (emulsifying agent) 1 part

The aqueous and oil phases are blended in equal volumes and emulsified by a spring-loaded piston mechanism similar to that illustrated by Himmelweit (1960).

Drakeol 6 VR is a light, white mineral oil produced by the Pennsylvania Refining Company. Arlacel A is mannide mono-oleate supplied by the Atlas Powder Company. Both are especially prepared for use with vaccines and are batch tested for toxicity in animals. Sterilization is by filtration to avoid the production of toxic by-products during heating. In addition to sterility tests, the final adjuvant vaccine preparations are tested for toxicity by the intra-peritoneal inoculation of mice.

In Part II of the trial volunteers received either 1.0 ml. of aqueous vaccine, "R", sub-cutaneously or 0.25 ml. (one dose) of "O", the adjuvant vaccine, by intramuscular inoculation into the belly of the triceps.

Mode of random selection

The vaccine which a volunteer received was determined by date of birth.

Part I

Those with odd-numbered days of birth (e.g., 1st, 3rd, 5th, etc. of the month) received "R" vaccine. Those with even-numbered days of birth received the placebo, "W".

Part II

Those with dates of birth in the first half of the

month (i.e., 1st - 15th) received adjuvant vaccine, "O", and those born in the second half of the month had aqueous vaccine, "R".

This method ensured that the volunteers would be randomly distributed between the vaccines in both parts of the trial and in relation of one part of the trial to another.

Syringes and needles

For inoculation:

For aqueous vaccines "R" and "W" 2 ml. disposable "Steriseal" (Shrimpton and Fletcher, Ltd.) syringes were used.

For the adjuvant vaccine "Mitrex" (Medical and Industrial Equipment, Ltd.) cartridge syringes with Luer fitting nose pieces were used. After use the detachable nose piece was boiled before re-use. The plunger thread was exposed so as to permit it to be engaged with the piston of the cartridge. This allowed withdrawal of the piston after the needle was inserted to avoid intra-vascular inoculation.

In Trial 1 "Steristar" (Smith and Nephew - Southalls, Ltd.) disposable needles of 23 gauge x 1 inch were used throughout.

For bleeding:

10 ml. "Steriseal" disposable syringes and "Scimitar" (Gillette Surgical, Ltd.) No. 1 disposable

needles of 21 gauge x 1½ inches were used.

Bleeding, inoculating and examining sessions

The times and places of sessions were arranged after consultation with the class representatives and the teaching departments who would be concerned. To allow for the successive examining sessions the initial inoculation session had to be on a Monday or a Tuesday.

The fourth and fifth year classes were inoculated and bled on Monday, November 12th, and the third year class on Tuesday, 13th. The first and second year classes were inoculated on the following Monday. A similar plan was adopted for Part II of the trial. Considerable effort was required to reconcile the trial with the diverse class programmes. All sessions were held during normal hours and close to where the students were working. Inevitably, however, some sessions were less convenient to attend than others.

Organisation of Sessions

Inoculation session:

Waiting volunteers were handed:-

(a) A Universal container with a blank label - for name and the date.

(b) A special record card (Demonstration 1) with space for the volunteer's name, address, and age in years.

(c) A small pink card - for the volunteer's name and date of birth.

After these had been filled in the volunteers were checked off a list at a desk and directed singly to one of three stations where they were inoculated with the vaccine, as determined by inspection of the pink card, and bled. Separate members of the medical staff bled and inoculated. The volunteers were not aware of the method of selection nor of the number of different vaccines. Most appeared to think that the different stations represented different vaccines. This impression seemed to hold even in the second part of the trial when the adjuvant vaccine was being administered with the cartridge syringe which was obviously different.

The record cards did not have any reference to the date of birth of the volunteer or of the vaccine, administered until after the relevant part of the trial was over. The pink cards with dates of birth on them were stored separately until the trial was over.

During inoculation sessions adrenaline and sal volatile were kept ready in case of need. In the case of the first year students there was a considerable incidence of fainting both while being bled and while waiting before any procedure was started. Later sessions were organised so that the volunteers did not watch others being bled and the problem was largely solved.

Checking sessions:

The volunteers' record cards were kept, class by class, in tickler boxes and were handed out to the volunteers to take to an examiner. Cards were marked, usually by an assistant, on the instructions of the examiner.

Considerable efforts were made to encourage full attendance at sessions for checking reactions and post cards were sent to remind defaulters of their obligations.

Sera

Blood specimens were allowed to clot overnight at 4° C. and the following day sera were separated and stored at -20° C.

At the initial bleeding of Part II of Trial 1, 1-2 mls. of blood was collected separately in 3.5 x 0.4 inch tubes for blood grouping and these were taken to the Blood Transfusion Service Laboratory soon after the session.

Trial 2 (November, 1962 - November, 1963)

Programme

A. Volunteers bled and inoculated with adjuvant influenza vaccines.

B. Volunteers examined for reactions to inoculation

i 2 days after inoculation

ii 7 days after inoculation

C. 28 days after inoculation volunteers were bled and examined.

D. 3 months after inoculation volunteers were bled and examined for reactions.

Volunteers

These were drawn from:-

A. First year students in the Faculty of Medicine at the Queen's University of Belfast. Students who were repeating the year and who had received vaccine during the previous winter's trial were excluded.

B. Nurses at the Royal Victoria Hospital and Royal Maternity Hospital who had volunteered to receive influenza vaccine.

As before, explanatory sheets and consent forms were circulated. The explanatory sheets were modified from the previous year (Appendix 1).

Vaccine

It was originally intended to test two adjuvant vaccines of identical components but of differing viscosity. However, the more viscous and more stable emulsion did not pass toxicity tests and all the volunteers received adjuvant vaccine precisely similar to "O" mentioned above but containing the following antigens:-

A/Singapore/1/57	1500 H.A.U.
A/England/1/61	500 H.A.U.
B/England/939/59	1000 H.A.U.
B/Taiwan/4/62	500 H.A.U. per dose

Note the influenza B component has been augmented by the addition of B/Taiwan/4/62. The dose of 0.25 ml. was administered into the upper portion of the long head of the triceps.

Syringes and Needles

For inoculation:

"Mitrex" syringes were used as before with either Johnson's No. 1 (Johnson's Ethical Plastics, Ltd.) 21 gauge 1½ inch or Scimitar (Gillette Surgical, Ltd.) No. 12, 23 gauge, 1 3/16 inch disposable needles. These were designed to cater for different dimensions of arm so that a longer needle was available to reach muscle adequately in the arms of fat people. In the event the nurses were all inoculated using the larger needle.

Bleeding:

10 ml. disposable syringes (Johnson's Ethical Plastics, Ltd.) and Scimitar No. disposable needles were used.

Sera:

Blood taken was partly allowed to clot overnight and the serum separated and stored as before. At each bleeding session 2 mls. of blood were kept in a separate tube and transferred to the Blood Transfusion Laboratory as soon as possible. In cases where there was some delay these bloods were kept at 4° C.

Bleeding, inoculating and examining sessions

The times and places of sessions were arranged with the first year class representative. Those for the nursing staff were organised by the Staff Medical Officer in the case of the three bleeding sessions. However, because the hours on duty and places of work of the nursing staff altered constantly it was often not possible to arrange sessions for all nurses and special arrangements had to be made for various groups. This was only possible because of the modified schedule of examining for reactions.

SECTION B REACTIONS

Materials and methods:

Trial 1

The record card (Demonstration 1) had spaces for recording the following reactions at the various checking sessions:-

1/ Local	Erythema	E
	Pain	P
	Tenderness	T
	Lymphadenitis	L
	"Other"	O
2/ Systemic	Malaise	M
	Fever	F
	Aches and Pains	A
	Gastric Symptoms (e.g. Nausea, Vomiting)	G
	Intestinal Symptoms (Diarrhoea)	I
	Headache	H
	Coryza	C
	Allergic manifestations	Al
	"Other"	O

Spaces "S" and "?" were for answers to questions.

Reactions were graded according to severity between 0 - 3 where this was applicable. Any reaction not covered by a code letter could be indicated in "Other" and explained on the back of the card.

INFLUENZA VACCINE TRIAL

Name: No.

Address: Age:

Vaccination Date: 1 2

Reactions: Local Systemic

	E	P	T	L	O	M	F	A	G	I	H	C	A	O	S	?
Immediate																
Hours																
Day 1																
2																
3																
to 7																
to 28																

Immediate															
Hours															
Day 1															
2															
3															
to 7															
to 28															

Arm Measurements:

	0	Hrs.	1	2	3	7	28	0	Hrs.	1	2	3	7	28
L														
R														

Bleedings. Dates

	1	2	3	4
A				
B				
O				

Demonstration 1. Card for recording reactions. Trial 1.

Examination for Reactions:

The Examiners:

The examination for reactions was carried out by members of the scientific and medical staff of the Department of Microbiology. Whenever possible they had assistants to write the results on to the cards. The system of recording and scoring of lesions was explained to the examiners before the trial began. However, these different examiners undoubtedly introduced an element of variation but it is likely that those who gave low scores were balanced by those who marked lesions higher. This factor could explain recordings that appear rather surprising on examining the cards.

The mode of examination:

The inoculated arm was exposed and examined for erythema, swelling or bruising. The volunteer would be asked how his arm was to ascertain subjective pain and the inoculated area was firmly palpated to detect induration, swelling or tenderness. The last was ascertained by questioning and observation. In addition a non-specific type of question such as, "And how are you?", was put, searching for evidence of systemic reaction.

An attempt was made to discover reasons for absence from defaulters but answers were not regularly obtained.

Measurement of arms:

An attempt was made to establish an objective index of reaction by measuring both arms before inoculation and at each session for examining reactions. However, it soon became clear that the degree of

error in measurement was so great and the degree of swelling so small that no useful purpose would be served by this examination and it was discontinued.

Questioning:

At the check 7 days after inoculation volunteers were asked whether the inoculation had interfered with work, play or social activities.

At the final check of a series (21 or 28 days after inoculation) volunteers were asked whether they thought that the inoculation was worth while if it kept influenza away. If further explanation was sought volunteers were asked whether the trouble they had had with the injection was, in their opinion, justified if they were kept free of influenza. Answers were recorded as "Yes", "No" or "Don't know" but in the first trial reasons for their decision were not requested. In addition, volunteers who had taken part in both phases of the first trial were asked 28 days after their second inoculation whether the first or second inoculation had been worse.

Significance tests:

In these results and in other sections of this study tests for significance were read at the 5% level. The chi-squared (X^2) test (performed according to Snedecor, 1946) was generally used, with grouping of results where necessary. Yates' correction was not applied. Where the chi-squared test would be inadmissable as, for instance, where the "Expected" value fell below 5 an Exact Probability was calculated.

Complete and Incomplete Records:

In each part of Trial 1 there were 7 sessions for the volunteers to attend. In both parts a considerable number of the volunteers did not attend all the sessions. In the first part only 73.9% had complete records and in the second only 78.1%. This display of independence introduced considerable complications into the analysis of the results.

To simplify, those who defaulted from one or more sessions could have done so:- 1/ Because the reaction caused was so severe as to either keep them away or to engender dislike or distrust of the trial.

2/ Because so little reaction occurred that they were not reminded of the inoculation or felt that it was not necessary to attend - despite exhortations.

3/ Because they forgot about the session or simply did not come to University that day for reasons of their own.

Observations on this point revealed that there was no significant association between the inoculation received and students defaulting. ($0.3>P>0.2$ and $0.95>P>0.9$ in Parts 1 and 2 of the trial respectively). Absenteeism was generally associated with the inconvenience of attending. For example, there was always a crop of defaulters on a Wednesday. This is the traditional half-day and some students simply fail to attend classes at all.

Inevitably, the existence of defaulters will introduce a source of error whether the results are included or not. If they are excluded one may be excluding reactions, if included they may weight the result unduly. In fact, in most cases the distribution of "maximum lesions"

in those with complete and incomplete records were similar and where dissimilar the incomplete records showed less severe reactions.

A compromise has been adopted in the presentation of results. For "Maximal lesion" tables results are shown separately and combined. In "Cumulative percentage" and "Daily record" tables the total of all observations, whether on people with complete or incomplete records, was used.

Exclusion of certain results:

After the assessment of reactions was over the actual inoculation received was deduced from the volunteer's date of birth. In a few cases this introduced problems.

In a few cases the date of birth written on the pink card was illegible and therefore it was uncertain how it had been interpreted or what inoculum had been given. In such cases the records of that subject were not included in the relevant part of the trial. In one case a student gave dates of birth which differed in regard to both day and month at the two different parts of the trial. However, on both pink cards the writing was clearly legible and therefore each was interpreted according to the face value. From the antibody results it seemed highly probable that another student received vaccine when he should have had placebo. The records of this volunteer were included in the group indicated by his date of birth.

RESULTS

Part 1

380 volunteers took part in the first part of the trial, 186 of

whom received placebo("W") and 194 vaccine ("R"). The numbers and sexes were distributed among the classes as follows:-

	Placebo	Vaccine R	Class
Male	33	30	
Female	9	14	1
		(Total 86)	
Male	30	29	
Female	7	15	2
		(Total 81)	
Male	28	24	
Female	10	11	3
		(Total 73)	
Male	27	27	
Female	5	7	4
		(Total 66)	
Male	25	31	
Female	12	6	5
		(Total 74)	

Local Reactions:

(a) **ERYTHEMA:**

Erythema around the inoculation site was scored as follows:-

1 indicated an area of erythema of up to 15 mm. in diameter

2 " " " " " " more than 15 mm. in diameter. This was occasionally as much as 50 mm.

3 would indicate widespread erythema about the arm. No records of "3" were made.

"Maximal Lesions":

Here the numbers and percentages of people are shown with their maximal lesion recorded during the trial:-

	Placebo (W)			Vaccine (R)		
	0	1	2	0	1	2
Males: Complete Records	102			78	9	18
Incomplete Records	40			31	1	5
Females: Complete Records	31			18	14	9
Incomplete Records	13			6	1	2
Total	186			133	27	34
	%					
Males: Complete Records	100%			74.2	8.6	17.2
Incomplete Records	100			83.7	2.7	13.6
Females: Complete Records	100			41.5	37.5	21.0
Incomplete Records	100			66.6	11.2	22.2
Total	100			68.5	13.9	17.6

"Cumulative Percentages:"

As signs and symptoms have duration, as well as incidence and grade of severity, this has to be indicated. Cumulative percentages are calculated on the total number of records of a particular grade during the first four examination sessions for reactions against the total number of examinations done during the four sessions. Only results from the first four examinations were included to avoid diluting the positive findings.

		Placebo W		Vaccine R	
		No.	%	No.	%
	Totals	685		714	
Grades	0	685	100	609	85.3
	1	-		62	8.7
	2	-		43	6.0
	3	-			

"Daily Record":

This records the number and grade of observations at each session. The total number of volunteers seen at each occasion varies inversely with the number of defaulters.

The mark (X) signifies the greater side if the distribution of

positive observations with one type of inoculation is significantly (see above) greater than the other.

	Placebo (W)				Vaccine (R)				
	2	1	0			0	1	2	
Session Hours			176			178	3		P =0.248
Day 1			180		X	140	25	17	P <0.0005
2			162		X	133	21	22	P <0.0005
3			167		X	158	13	4	P <0.0005
7			177			187	2	1	P =0.248
21			181			192			

It will be seen that women showed erythema to a greater extent than men (53.8% as against 23.3%). Erythema was maximal on the first and second days after inoculation and was still present in three persons a week after inoculation. This reaction was seen only in association with vaccine.

(b) PAIN:

This was essentially judged on subjective grounds and people differ in their reaction to discomfort. It was assessed by asking volunteers about their arms. Sometimes they moved their arms about to see if they felt the inoculation. Pain was scored as 0, ½ (minimal), 1 (slight), 2 (moderate) and 3 (severe). The "½" scoring was introduced to cover those who were merely aware of having been inoculated. A grade of 3 would have indicated marked discomfort on using the arm. No scores of

3 were given. Pain was differentiated from tenderness as being hurt experienced at rest or with use but without contact.

"Maximal Lesions"

	Placebo W				Vaccine R			
	0	½	1	2	0	½	1	2
Males								
Complete Records	80	10	11	1	82	11	11	1
Incomplete "	35	2	3	-	27	5	4	1
Females								
Complete Records	27	1	3	-	25	8	9	1
Incomplete "	10	1	2	-	7	2	-	-
Total	152	14	19	1	141	26	24	3
	%							
Males								
Complete Records	79.3	9.8	9.9	1.0	78.1	10.5	10.5	0.9
Incomplete "	87.5	5.0	7.5	-	73.0	13.5	10.8	2.7
Females								
Complete Records	87.0	3.2	9.8	-	58.0	18.7	21.0	2.3
Incomplete "	77.0	7.7	15.3	-	77.8	22.2		
Total	81.8	7.5	10.2	0.5	72.4	13.5	12.4	1.7

"Cumulative Percentages"

	Placebo W		Vaccine R	
	No.	%	No.	%
Totals	685		714	
0	648	94.6	640	89.6
½	17	2.5	36	5.0
1	19	2.8	34	4.8
2	1	0.1	4	0.6

"Daily Record":

	Placebo W					Vaccine R					
	2	1	½	0			0	½	1	2	
Hours	1	15	12	148			151	11	18	1	0.7>P>0.6
Day 1		4	5	171		X	150	18	11	3	0.01>P >0.005
2				162		X	169	4	3		P = 0.015
3				167			170	3	2		P = 0.061
7				177			190				
21				181			192				

It will be seen that a few hours after inoculation the pain caused by both placebo and vaccine were similar. However, on the two days following there were significantly more complaints with the vaccine. Complaints had returned to the level of chance distribution by the third day although the only complaints occurred with the vaccine.

There was no significant difference between records for men and women over all (0.2>P>0.1), but in response to Vaccine "R" women suffered more than men (0.05>P>0.025).

(c) TENDERNESS:

The presence and grade of tenderness was deduced by the response to questioning and firm palpation. Occasionally, when the general area of inoculation was not tender a record was made when tenderness was elicited by direct pressure on the site of the needle entry. Tenderness, again, was mainly a subjective response but less so than pain, as the reaction to pressure could be observed. This was the commonest sign of reaction as tenderness persisted longer than a feeling of pain or discomfort. However, no one was severely afflicted, as with primary vaccinia lesions or with typhoid inoculation and no records of grade "3" were made.

"Maximal lesions"

	Placebo W				Vaccine R			
	0	½	1	2	0	½	1	2
Males								
Complete records	98	4			69	18	16	2
Incomplete records	39	-	1		24	10	3	-
Females								
Complete records	25	2	4		17	7	18	1
Incomplete records	11	-	1	1	4	5	-	-
Total	173	6	6	1	114	40	37	3

	Placebo W				Vaccine R			
	0	½	1	2	0	½	1	2
	%							
Males								
Complete records	96.1	3.9			65.7	17.2	15.2	1.9
Incomplete records	97.5		2.5		64.9	27.0	8.1	-
Females								
Complete records	80.5	6.5	13		39.5	16.2	42.0	2.3
Incomplete records	84.6		7.7	7.7	44.2	55.8	-	-
Total	93.0	3.2	3.2	0.6	58.5	20.7	19.2	1.6

Women showed significantly more reactions than men even considering both inoculations overall ($P < 0.0005$).

Cumulative percentages.

	Placebo W		Vaccine R	
	No.	%	No.	%
Total	685		714	
0	672	98.1	589	82.5
½	7	1.0	66	9.2
1	6	0.9	56	7.9
2			3	0.4

The cumulative percentages indicate the marked difference between the two inocula.

"Daily Record"

	Placebo (W)					Vaccine (R)					
	2	1	½	0			0	½	1	2	
Hours		1	4	171		X	166	7	7	1	0.05 > P > 0.025
Day 1		3		177		X	138	23	21		P < 0.0005
2		1	1	160		X	142	18	14	2	P < 0.0005
3		1	2	164		X	143	18	14		P < 0.0005
7		2	2	173			185	2	3		
28	1			181			191		1		

Records of tenderness show a marked difference between the inocula from a few hours after inoculation until after the third day. Tenderness at a week, however, seemed to be a function of the injection itself rather than the vaccine.

(d) INDURATION AND SWELLING:

Induration and swelling were recorded under "Other" and were not graded for severity. However, it is clear that these were not found in many people and it shows that although swelling was only found in association with the vaccine the attempt to use it as an index was found to fail.

Total records.

Hours	W	R
1 Day		Induration 2 Swelling 1
2		Induration 5
3		Induration 6
7		
21		

(e) BRUISES:

Bruising at the site of inoculation occurred with injections of both placebo and vaccine. Bruises varied considerably in size from small circumscribed areas less than 5 mm. across, around the needle mark, upwards. Some clearly stemmed from the puncture of cutaneous blood vessels during inoculation.

No specifications were laid down governing bruises so that the grading of them was a matter of individual judgment.

"Daily record"

	Placebo W						Vaccine R				
	2	1	½	0			0	½	1	2	
Hours			1	175			178		1	1	
Day 1	1	5	4	170			176	2	3		0.2>P>0.1
2			3	159			170	1	4		
3		3	4	160		X	156	5	13		0.05>P>0.025
7			2	175			186		3		
21				182			192				

It is possible that the large incidence of bruises recorded on the third day may be partly due to the interpretation of fading and discoloured erythema as a bruise. With the exception of this day the incidence of bruises is rather evenly spread between the two inocula.

(f) LYMPHADENITIS:

Two volunteers receiving vaccine reported a feeling of mild fullness in the regional axillary nose area on the day after inoculation. On the same day one student in the placebo group is recorded as having "axillary pain". This might have been a similar complaint.

Systemic Reactions:

(a) ALLERGIC REACTIONS

No immediate allergic manifestations occurred. Other disorders of a possible allergic nature that occurred during this part

of the trial are listed below.

	Placebo W	Vaccine R
Hours		
Day 1	1 case "itching"	
2		1 case "itching"
3	1 case "itching"	1 case "itching"
7	1 case urticaria	1 case urticaria
		1 case transient rash
21		

There was no indication on the record cards whether any of the cases of "itching" were anything more than localized itching at the site of inoculation. Both volunteers who had urticaria suffered regularly from this complaint. In the case who had vaccine the urticaria was mild and localized, but in the other the complaint was more generalized and involved different parts of her body over a period of several days.

(b) CORYZA:

Under this heading are included all non-specific mild upper respiratory affections such as colds, coughs and sore throats where there was no indication of fever.

	Placebo (W)	Vaccine (R)
Hours	1	
Day 1	4	3
2	1	
3	1	2
7	5	1
21	15	16

These listed under Day 7 or Day 21 include all those colds suffered in the period since the previous inspection. For the last two weeks of observation this represents an incidence of just over 8%.

(c) MALAISE AND "'FLU":

This section included all reports of fever or shivers and, probably, would include nearly all these responses described, non-specifically, in the literature as "systemic reactions".

	Placebo (W)	Vaccine (R)
Hours		
Day 1	3	
2		2
3		1
7		
21	3	4

Some of the complaints listed here were very non-specific and include, on the day following inoculation, a case of headache. Among those receiving vaccine at 21 days one record was of malaise and aches after playing rugby.

Miscellaneous:

A number of varied conditions were encountered. In the group who were given placebo there were two cases of migraine, one, 1 day and the other, 7 days after inoculation, one attack of paroxysmal tachycardia which came on during a game of squash 2 days after inoculation and at 21 days there was one report each of "gastric 'flu", Bornholm disease and food poisoning. A female student was admitted to hospital with pneumonia about 2 weeks after inoculation with placebo.

Amongst those who received vaccine there was a report of one volunteer with diarrhoea 1 day after inoculation and another case of diarrhoea and vomiting between the 7th and 21st days.

General Responses:

In assessing the answers to the question as to whether the vaccine interfered with normal activities, replies that indicated that the volunteer found sleeping on the inoculated arm uncomfortable, or that he was glad that he had not been inoculated in the arm which he used for writing, were recorded as signifying interference with normal activities. Five complaints were received; three from those receiving placebo and two from the vaccine group.

Trial 1 Part 2

Inoculations in the second part of the study were carried out in the first half of January, 1963, two months after those of the first part. There were fewer volunteers for the second part than for the first. 342 took part and 267 had complete records. Three students joined the trial for the first time, having not received any vaccine previously, and 41 left. The records of these 41 volunteers were studied to see whether their reactions had been worse than the average.

Of this 41, 26 had had placebo and 15 vaccine. When the incidence of various reactions in these were compared with those shown by the volunteers who remained in the trial no significant differences were found. The probabilities that the reactions shown by these falling out are within the chance distribution are:-

Reaction	Vaccine	
	W	R
Erythema		$0.9>P>0.8$
Pain	$0.9>P>0.8$	$0.6>P>0.5$
Tenderness	$P = 0.088$	$0.7>P>0.6$

(In some of the probabilities chi-squared was calculated despite rather low values in one cell of the table.)

Of these in the second part of the trial 158 were given the adjuvant vaccine "O" and 184 received 1.0 ml. of the aqueous vaccine "R".

The numbers and sexes were divided among classes as follows:-

	Adjuvant "O"	Aqueous "R"	Class
Male	20	32	1
Female	5	14	
		(Total 71)	
Male	25	28	2
Female	11	11	
		(Total 75)	
Male	17	30	3
Female	9	12	
		(Total 68)	
Male	25	26	4
Female	4	7	
		(Total 62)	
Male	30	18	5
Female	12	6	
		(Total 66)	

Local Reactions

(a) ERYTHEMA:

The degree of erythema was scored as in the first part of the trial.

"Maximal Lesions"

	Adjuvant O				Aqueous R			
	0	1	2	3	0	1	2	3
Males:								
Complete Records	86				94	4	7	
Incomplete Records	33				29	1		
Females:								
Complete Records	35				30	4	6	
Incomplete Records	3		1		8	1		
Total	157		1		161	10	13	
	%							
Males:								
Complete Records	100				89.5	3.8	6.7	
Incomplete Records	100				96.7	3.3		
Females:								
Complete Records	100				75	10	15	
Incomplete Records	75		25		88.9	11.1		
Total	99.4		0.6		87.5	5.4	7.1	

"Cumulative Percentages"

	Adjuvant "O"		Aqueous "R"	
	No.	%	No.	%
Total	587		686	
0	586	99.8	653	95.2
1			17	2.5
2	1	0.2	16	2.3
3	-	-	-	-

"Daily Record"

	Adjuvant "O"				Aqueous "R"				
	2	1	0			0	1	2	
Hours			146			175			
1			155		X	170	10	7	P < 0.0005
2	1		141		X	162	6	5	0.01 > P > 0.005
3			144			156	1	4	P = 0.062
7			154			182			
28			158			184			

Only one volunteer receiving adjuvant vaccine showed erythema. The incidence of erythema with 1.0 ml. of aqueous vaccine (R) in the second part of the trial was significantly less than with 0.5 ml. of the same vaccine in the first part ($P < 0.0005$). This did not seem to be associated with previous experience because if the incidence of

erythema in the second part of the trial is examined in relation to the inoculum received in the first:-

First Part		Erythema in Second Part	
Inoculum	No.	No.	%
No vaccine or placebo "W"	88	12	13.6
Vaccine "R"	95	11	11.6

This distribution could have occurred by chance ($0.7>P>0.6$)
(The divergence in numbers of volunteers is because the inoculum received by one student in the first part is uncertain.)

(b) PAIN:

The system of scoring was the same as in the first part of the trial.

"Maximal Lesions"

	Adjuvant "O"				Aqueous "R"			
	0	½	1	2	0	½	1	2
Males:								
Complete Records	48	17	19	2	32	31	20	3
Incomplete Records	24	7	1	1	11	18	2	2
Females:								
Complete Records	11	14	10		12	12	10	1
Incomplete Records	2	1	1		2	2		
Total	85	39	31	3	57	63	32	6

	Adjuvant "O"				Aqueous "R"			
	0	½	1	2	0	½	1	2
	%							
Males:								
Complete Records	55.8	19.8	22.1	2.3	47.6	25.7	22.9	3.8
Incomplete Records	72.7	21.2	3.0	2.0	40.0	40.0	16.7	3.3
Females:								
Complete Records	31.4	40.0	28.6		35.0	20	32.5	12.5
Incomplete Records	50	25	25		66.7	11.1	22.2	
Total	53.8	24.7	19.6	1.9	44.6	26.1	23.9	5.4

"Cumulative Percentages"

	Adjuvant "O"		Aqueous "R"	
	No.	%	No.	%
Total	587		686	
0	464	79.1	534	77.8
½	73	12.4	87	12.7
1	47	8.0	55	8.0
2	3	0.5	10	1.5
3				

"Daily Record"

	Adjuvant "O"						Aqueous "R"				
	2	1	½	0			0	½	1	2	
Hours	1	5	22	118		X	104	44	22	5	P<0.0005
Day 1	1	22	23	109			120	26	27	4	0.8>P>0.7
2	1	15	18	108	X		152	14	6	1	0.025>P>0.01
3		5	10	129	X		158	3			0.005>P>0.001
7				154			182				
28				158			184				

Women were more affected than men overall (0.005>P>0.001). Whether the volunteers had had vaccine or placebo in the first part had no effect upon the complaints in the second part (with "O", 0.9>P>0.8; with "R", 0.7>P>0.6).

The aqueous vaccine gave rise to symptoms earlier but these persisted longer with the adjuvant vaccine.

(c) TENDERNESS:

Tenderness was frequently elicited. The mode of examination and of scoring was as in part 1 of the Trial. However, examiners were warned to palpate for the presence of cysts or sterile abscesses, in particular, in case any were found in association with the adjuvant vaccine.

"Maximal Lesions"

	Adjuvant "O"				Aqueous "R"			
	0	½	1	2	0	½	1	2
Males								
Complete Records	32	31	20	3	25	48	27	5
Incomplete Records	11	18	2	2	9	15	5	1
Females								
Complete Records	12	12	10	1	6	13	18	3
Incomplete Records	2	2	-	-	1	4	3	1
Total	57	63	32	6	41	80	53	10
	%							
Males								
Complete Records	37.2	36.0	23.3	3.5	23.8	45.7	25.7	4.8
Incomplete Records	33.3	54.5	6.1	6.1	30.0	50.0	16.7	3.3
Females								
Complete Records	34.3	34.3	28.6	2.8	15.0	32.5	45.0	7.5
Incomplete Records	50	50			11.1	44.4	33.3	11.1
Total	36.1	39.9	20.3	3.7	22.3	43.5	28.8	5.4

"Cumulative Percentages:"

	Adjuvant "O"		Aqueous "R"	
	No.	%	No.	%
Total	587		686	
0	361	61.5	371	54.1
½	167	28.4	217	31.6
1	51	8.7	86	12.5
2	8	1.4	12.	1.8
3				

"Daily Record"

	Adjuvant "O"					Aqueous "R"				
	2	1	½	0		0	½	1	2	
Hours		7	38	101		107	47	16	3	0.2>P>0.1
Day 1	2	23	45	85	X	69	61	43	4	0.01>P>0.005
2	5	14	47	76		83	64	21	5	0.7>P>0.6
3	1	7	37	99		112	43	6		0.8>P>0.7
7			2	152		174	8			P = 0.134
28				158		184				

There was no significant difference between the results of the two vaccines except on the first day when tenderness was more marked with the aqueous vaccine. The Cumulative Percentages seem to indicate that the effect of the aqueous vaccine was more marked when the first three days are considered. However, some degree of tenderness was clearly very common. The greater effect on women rather than men was, in this case, only just discernable ($0.05>P>0.025$).

(d) SWELLING AND INDURATION:

Once again, these signs were only present in those getting aqueous vaccine. This could probably be ascribed to the site of inoculation as well as to the different amounts of antigen.

Total records of swelling and induration:

	Adjuvant 0	Aqueous R
Hours		
Day 1		Induration 4 Swelling 2
2		Induration 1 Swelling 2
3		Induration 2 Swelling 2
7		Induration 1
28		

It will be noticed that induration at the site of inoculation was still present a week after inoculation.

(e) BRUISING:

Bruising at the site of inoculation occurred as in the first part of the trial, but occurred more frequently with the adjuvant vaccine. However, on a day-to-day basis the difference was not significant.

	Adjuvant O					Aqueous R				
	2	1	½	0		0	½	1	2	
Hours		2	1	143		173	1	1		
Day 1	1	5	2	147		174	2	1		0.1>P>0.05
2	1	4	3	134		169	2	2		0.2>P>0.1
3		4	2	138		157	1	3		
7		2	1	151		180	1	1		
28				158		184				

(f) LYMPHADENITIS:

One case of axillary lymphadenitis was recorded on the 3rd day. The volunteer had received aqueous vaccine.

Systemic Reactions

(a) ALLERGIES:

Again no immediate allergic affects were noticed. All the cases with symptoms of a possibly allergic nature occurred in the group receiving aqueous vaccine.

"Total reports"

	Adjuvant "O"	Aqueous "R"
Hours		1 urticaria
Day 1		1 "itching"
2		
3		
7		
28		1 rash

The "urticaria" above consisted of a single wheal near the elbow. The record card had a suggestion that this was caused by scratching. Both the volunteers who (after one inoculum or another) had urticaria in the first part of the trial received adjuvant vaccine in the second without any allergic symptoms.

The volunteer who reported a rash occurring between the 7th and 28th day apparently had had a similar lesion in the past, not associated with vaccine.

(b) CORYZA:

	Adjuvant "O"	Aqueous "R"
Hours	2	5
Day 1	1	4
2	4	2
3		1
7	1	4
28	10	21

Colds, coughs and sore throats appeared to be more common among those given aqueous vaccine.

(c) MALAISE AND "'FLU":

As in the first part this group includes headache (except migraine) and all non-specific conditions where fever or "shivers" were reported.

	Adjuvant "0"	Aqueous "R"
Hours		1 Headache
Day 1		1 Sore throat & shivers 1 Headache 1 Dizziness & malaise
2		1 Malaise
3		1 Malaise
7		2 "Flu"
28		2 "Flu"

The student who felt dizzy and unwell on the first day after inoculation felt the same the next day and went to bed on the third day. (This is recorded on all three days.) Symptoms were quite non-specific and on examination nothing abnormal was detected. A similar incident had occurred some years previously in association with a cold. This student had had no reaction to 0.5 ml. of vaccine in the first part of the trial.

(d) MISCELLANEOUS:

Among those receiving "R" vaccine one volunteer had migraine the

afternoon after inoculation, one had sinusitis on the second day and a third had diarrhoea and vomiting three days after inoculation. One case of possible glandular fever occurred between the 7th and 28th days.

In the group given adjuvant vaccine, "O", there were cases of "gastric 'flu" recorded. One occurred on the 7th and the other near the 28th day.

Of more direct interest, a painless, subcutaneous lump was found near the olecranon of an African student 3 days after she had received adjuvant vaccine. The student asserted that this had followed a yellow fever inoculation in that site and this had preceded the influenza vaccine. This lump disappeared two months after the influenza vaccine inoculation. In addition, one student developed symptoms of radial nerve irritation following the intramuscular inoculation. These symptoms of paraesthesiae disappeared in three days. On examination there was no altered sensation, nor any objective weakness.

General Responses:

No volunteer claimed that the inoculation had interfered with work, play or social activities. Apparently, the student who had three days of non-specific malaise was disinclined to attribute this to the inoculation.

In both parts of the trial, at the time of the final examination, the students were asked whether they thought the inoculation was worth while.

"Was the inoculation worth while?"

	First Part		Second Part	
	Placebo (W)	Aqueous Vaccine (R)	Adjuvant Vaccine (O)	Aqueous Vaccine (R)
		0.5 ml.		1.0 ml.
	(186)	(194)	(158)	(184)
Yes	80%	84.3%	67.7%	67.9%
No	3.8%	4.6%	8.2%	11.4%
Don't know	10.2%	7.7%	10.8%	9.8%
No answer recorded	6.0%	3.1%	13.3%	10.9%

There was no significant difference between the answers for placebo and 0.5 ml. of aqueous vaccine in the first part ($0.5>P>0.3$) nor between adjuvant and 1.0 ml. of aqueous vaccine in the second part ($0.8>P>0.7$). There is a significant difference between the answers for the two parts of the trial even when those cards without answers are eliminated ($0.025>P>0.01$).

The reasons for negative answers were not systematically recorded. Some said that vaccination was not worth while because they never got influenza or got it so rarely. One is recorded as saying that he would prefer an attack of influenza to the vaccine.

Where it was applicable volunteers were asked to compare the two inoculations they received. This was asked at the final examination 28 days after the second injection. Four groupings were possible.

First Part	Placebo				Vaccine "R"			
Second Part	Adjuvant "0"		Aqueous "R"		Adjuvant "0"		Aqueous "R"	
The 2nd inoculation was								
Better	21	28.8%	18	20.45%	24	28.9%	32	33.7%
Worse	29	39.7	39	44.3	30	36.1	34	35.8
Don't know	9	12.3	13	14.8	15	18.1	18	18.9
No answer recorded	14	19.2	18	20.45	14	16.9	11	11.6

These figures do not reveal significant preference for one vaccine or another ($0.7>P>0.6$) or even for placebo over vaccine ($0.2>P>0.1$) even when non-committal answers are excluded.

In October 1963 an appeal was made to the volunteers who had taken part in the second part of the trial to come forward to be bled once more to furnish information regarding antibody titres. 301 of the 342 were bled. 143 had received adjuvant vaccine and 158 aqueous vaccine. At this time the students were questioned about reactions and their arms were examined for cysts, thickenings of the muscle or sterile abscesses that might have followed adjuvant vaccine. No such lesions were detected.

Trial 2

Several modifications were introduced into the second trial.

1/ Because of the absence of a control vaccine and the relatively

small numbers involved it was felt that less information concerning reactions could be obtained. Therefore less stress was placed upon this aspect of the trial. Fewer examinations were made but more questions were asked and the record card redesigned to allow the answers to be more easily recorded (Demonstration 2).

Space for the recording of bruises and swelling was also provided (under "B" and "S") but several other columns were deleted.

2/ With the reduced number of examining sessions the problem of incomplete records did not assume the same importance. However, it was not always possible to see all the volunteers on the correct day. The varied programme of duty of the nursing staff constituted a particular difficulty as during the trial some were transferred to different hospitals and others went on night duty or on leave. One female medical student missed the examination at 7 days and a male student missed that at 28 days.

3/ In order to avoid the possibility of inoculations involving the radial nerve and at the same time to retain the advantages of the triceps site for assessing reactions, a member of the Anatomy Department was consulted (Dr. W.R.M. Morton) and it was decided to inject into the upper part of the long head of the triceps.

INFLUENZA VACCINE TRIAL 1963-1964

Name: No.

Address: Age(yrs)

	No	Yes	When last
Previous influenza vaccine			

Reactions

	E	P	T	S	B	O	M	F	C	Al	O	
Day 2												
Day 7												

	No	Yes	Details 1	2	3	
Hindrance						
Loss of activity						

	Yes	No	P			
Repeat						

Immunity	
Other	

Day 28	SW	Re	Resp D	
Yes				
No				
Day 90				
Yes				
No				

Demonstration 2: Card used for recording reactions during second trial.

Numbers:

Volunteers were distributed in the following numbers:-

Source	Sex	Numbers
First year students	Male	45
	Female	23 (68)
Nursing staff	Female	29 (97)

Materials and methods:

Examination:

The examination for reactions was carried out in the same manner as in the previous trial but on the 2nd and 7th days after inoculation.

At the 7th day volunteers were asked:

1/ "Did your arm trouble you?" If the answer was in the affirmative an indication of the grade (slight, moderate or severe) was obtained and recorded.

2/ "Did the inoculation cause you to miss any work, play or social activities?" Space was left for the answer and any details.

3/ "Would you have this vaccine again if it kept influenza away?"

For the event of negative answers space was left for reasons.

Two reasons for refusing further inoculation were listed on the card; one being "pain" (i.e., the injection caused too great a reaction) and "immunity" (i.e., they never get influenza).

At the 28th day and at three months the sites of inoculation were examined for residual swellings or cysts and volunteers were asked about illness in the intervening period.

Presentation of results:

As the conditions of the trial are different the types of tables that were used in the first trial would not be suitable for the second.

For each day of examination the distribution of reactions in grades is shown. The results are shown both for each category of volunteer and for the whole population. This is compared with the distribution for adjuvant vaccine in the previous trial on the same day after inoculation. Percentages are shown in brackets.

RESULTS:

Local Reactions:

(a) ERYTHEMA:

	Students		Nurses	Total	
	Male	Female			"O" Vaccine
(Day 2)					
0	44 (97.8)	22 (95.7)	27 (93.1)	93 (95.9)	141 (99.3)
1	1 (2.2)	1 (4.3)	1 (3.45)	3 (3.1)	
2			1 (3.45)	1 (1.0)	1 (0.7)
3					

No erythema was observed 7 days after inoculation. Differences in the incidence of erythema between "0" vaccine and that used in Trial 2 could be due to chance (P = 0.161).

(b) PAIN:

The grade for this symptom was determined in the same way as in the previous trial.

	Students		Nurses	Total	
	Male	Female			"0" Vaccine
(Day 2)					
0	31 (68.9)	19 (82.6)	12 (41.4)	62 (63.9)	108 (76.0)
½	8 (17.8)	2 (8.7)	10 (34.5)	20 (20.6)	18 (12.7)
1	6 (13.3)	2 (8.7)	6 (20.7)	14 (14.4)	15 (10.6)
2			1 (3.4)	1 (1.0)	1 (0.7)
3					
(Day 7)					
0	45	22	28 (96.6)	95 (99.0)	154
½					
1			1 (3.4)	1 (1.0)	
2					
3					

The overall results on the 2nd day are comparable with those found with the adjuvant vaccine "0" in the first trial ($0.2 > P > 0.1$). However. when the nurses are considered they show significantly more reactions than the students ($0.005 > P > 0.001$) and than those receiving vaccine "0" ($P < 0.0005$).

The great majority had no complaint at the examination at 7 days.

(c) TENDERNESS:

	Students		Nurses	Total	"0" Vaccine
	Male	Female			
(Day 2)					
0	26 (57.8)	12 (52.2)	17 (58.6)	55 (56.7)	76 (53.5)
½	7 (15.5)	7 (30.4)	9 (31.0)	23 (23.7)	47 (33.1)
1	12 (26.7)	3 (13.0)	0	15 (15.5)	14 (9.9)
2		1 (4.4)	3 (10.3)	4 (4.1)	5 (3.5)
3					
(Day 7)					
0	45	22	26 (89.7)	93 (96.9)	152 (98.7)
½			1 (3.4)	1 (1.0)	2 (1.3)
1			2 (6.9)	2 (2.1)	
2					
3					

The incidence of tenderness on the second day was comparable within the three categories of volunteer ($0.9>P>0.8$) and slightly, but no significantly, lower than found with "0" vaccine ($0.2>P>0.1$). However, it will be seen that the proportion graded as "1" or "2" was greater in the second trial than in the first.

(d) BRUISING:

The severity of bruises was scored from ½ - 3 according to the examiner's opinion.

	Students		Nurses	Total	"0" Vaccine
	Male	Female			
Day 2					
0	42 (93.3)	20 (87.0)	21 (72.4)	83 (85.6)	134 (94.4)
½		2 (8.7)		2 (2.1)	3 (2.1)
1	2 (4.4)	1 (4.3)	5 (17.3)	8 (8.2)	4 (2.8)
2	1 (2.2)		3 (10.3)	4 (4.1)	1 (0.7)
3					
Day 7					
0	45	22	22 (75.8)	89 (92.7)	151 (98.1)
½			1 (3.5)	1 (1.0)	1 (0.6)
1			5 (17.2)	5 (5.2)	2 (1.3)
2			1 (3.5)	1 (1.0)	
3					

Here again the significant difference between the "0" vaccine and the current trial ($0.025 > P > 0.01$) was caused by the high incidence of bruises found in the nurses. The student group showed only a slight excess in incidence and this was not beyond the bounds of chance occurrence ($0.4 > P > 0.3$). The persistence of the nurses' bruises was surprising.

(e) SWELLING:

Visible swelling was seen in the case of three nurses and three medical students (two of these being females). Unlike the bruising this did not persist to 7 days.

(f) MISCELLANEOUS:

One nurse reported mild regional lymphadenitis 2 days after inoculation and one felt her arm ache when she lifted it. At 28 days another nurse reported that the inoculated arm still felt stiff. On examination nothing was found locally nor was there limitation of movement.

(g) SUBCUTANEOUS NODULES:

At the examination at 28 days after inoculation, four volunteers, two nurses and two female medical students, were found to have subcutaneous nodules at the site of inoculation. These nodules were fairly well defined, separate from the skin and the muscle and were slightly tender on pressure. They were all first noticed by the volunteers about 2 weeks after inoculation. The nodules varied in size from about 2 x 1.5cms. to 1 x 1 cm.

These volunteers were followed up at intervals of a month. The nodule, in one nurse, had disappeared by two months after inoculation. In the other three volunteers the nodules regressed slowly. Six months after inoculation the lesions were barely detectable in two cases and very small in the third. Some residual tenderness was still present in one case.

Systemic Reactions:

With the possible exception of a localized "rash" at the inoculation site reported at the 28th day no allergic manifestations were observed.

In the immediate post-inoculation phase one female medical student reported feeling faint for ½ - 3 hours after inoculation and a nurse reported a cold starting the same evening. Rather later were two complaints of nausea, with vomiting in one case, starting 26 - 36 hours after inoculation. In one case, home contacts who had not been inoculated also had the same symptoms. One nurse reported the onset of a febrile cold 36 hours after inoculation. In addition there were these others:

	Students		Nurses
	Male	Female	
Day 2	1 cold		
Days 2 - 7	1 cold		2 colds 1 faint during hockey
Days 7 - 28		1 "gastric 'flu" 2 "'flu"	5 colds 1 "'flu" 1 bronchitis
Days 28 - 90		1 cold	3 colds 1 bronchial catarrh 1 "'flu"

Although there are a number of complaints no clear-cut pattern of systemic reactions emerges. The nurses seemed to have more colds than the students. The nurses might be more exposed to such infections in

the course of their occupation.

General Responses:

On the 7th day after inoculation all volunteers were asked:-

(1) Whether their arm troubled or hindered them. Of 96 questioned, 87 (90.6%) answered "No", and 9 (9.4%) answered "Yes". All these 9 were females and 8 were nurses. All graded the amount of nuisance as "slight" (Grade 1).

(2) Whether the inoculation caused any loss of work, play or social activity. No one reported that activities had been curtailed.

(3) Whether they would have the inoculation again if it kept influenza away. 94 (97.9%) said they would, 2 (2.1%) said they would not. Both these were male students and the reason given in both cases was that they never got influenza.

Reactions

Animal Experiments

Because sub-cutaneous nodules developed in a proportion of the volunteers in the second trial after adjuvant a number of experiments were carried out on monkeys to examine the histology at the site of inoculation of adjuvant and aqueous vaccines. Many studies of the reaction about oily adjuvants have included Mycobacterium species in the adjuvant, have used reagents that would not be acceptable for use with current vaccines or have employed large volumes of inoculum (Friedewald, 1944, Freund, Thomson, Sommer, Walter and Pisani, 1948, Moore, Lamm, Lockman and Schoenberg, 1963).

Rhesus monkeys (Macaca mulatta), which were being used as a source of kidneys for tissue culture, were used and they were inoculated, sometimes at several sites during the course of the experiment, with 0.25 ml. doses of adjuvant vaccine, as used in the second trial, or 1.0 ml. of aqueous vaccine "R". The injections were timed so that when the second kidney was removed the heavily anaesthetized animals were perfused through the heart, first with isotonic saline and then with 10% formol-saline. Specimens were taken for histology as required and fixed in 5% formol-saline. Blocks were cut and processed and paraffin sections were stained with haematoxylin and eosin.

The stained sections were prepared by Mr. J.A. Reid F.I.M.L.T., of the Histopathological Laboratories of the Royal Victoria Hospital.

Adjuvant Vaccine

In the case of the adjuvant vaccine the pattern at the site of inoculation evolved slowly and a number of specimens were studied.

1. Intramuscular inoculation.

Two days after inoculation the inoculum was seen to have spread between the muscle bundles forcing them apart. The oily material, , naturally, disappeared during processing of the preparations and left spaces. However, a patchy cellular reaction was just appearing in the connective tissue around the drops of inoculum. Eosinophils, macrophages and lymphocytes were the predominant cell types and polymorphs were only rarely seen (Figure 1).

In a specimen taken 7 days after inoculation, the cellular reaction around the oil droplets, scattered over an area of 8 x 4 mm., had not progressed much but localised areas of degenerating muscle fibres were seen (Figure 2). As this degeneration was not observed all around the droplets it was probably related to pressure rather than toxic effects of the vaccine.

By 14 days a fully developed cellular reaction was present. The drops of inoculum were surrounded by a

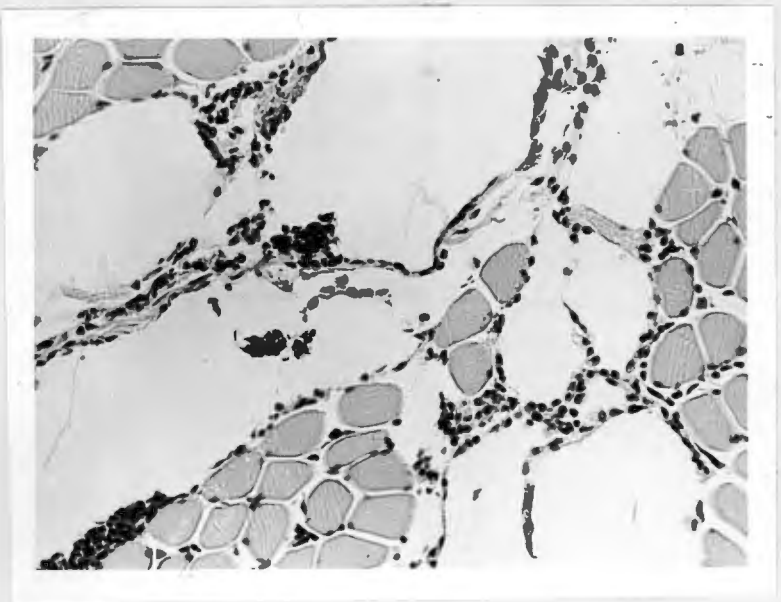


Figure 1. Two days after inoculation of adjuvant vaccine intramuscularly. (x 240)

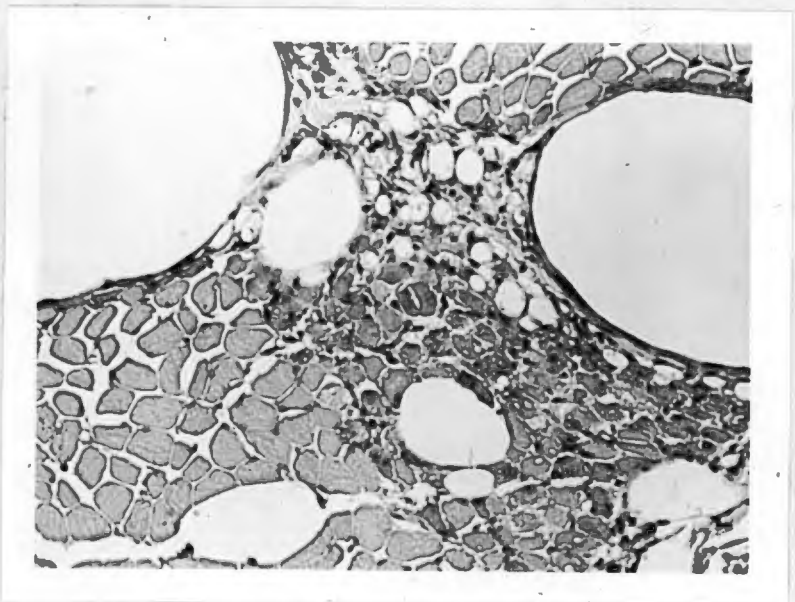


Figure 2. Degeneration of muscle fibres. Seven days after inoculation of adjuvant vaccine. (x 240)

layer of eosinophils and lipoid-filled macrophages (Figure 3). Plasma cells occurred in localised areas rather than diffusely. Fibrosis was commencing between the areas of reaction around the drops of oil. Foreign body giant cells were seen and occasionally were prominent (Figure 4).

A section of a specimen taken 52 days after inoculation shows that although the oil droplets are still present, if reduced in size, the reaction has largely died down and fibrosis is prominent (Figure 5).

2. Sub-cutaneous inoculation:

As young monkeys lack a well-developed sub-cutaneous tissue it is more difficult to reproduce the picture seen in man. However, by 14 days after inoculation a palpable nodule was present under the skin and on section measured 10 x 6 mm. Histologically, the picture was similar to that seen in the intramuscular site with the oil droplets surrounded by a mononuclear cell reaction but was in a more localised area (Figure 6).

By 52 days a similar lesion presented a firm nodule. Microscopically, the area had shrunk and the reaction around the persistent oil drops had receded. The sub-cutaneous tissue was swollen with fibrous tissue which was much infiltrated with lymphatic channels (Figure 7).

3. Regional nodes:

In two cases the regional nodes were examined two weeks after inoculation. In one of these the nodes

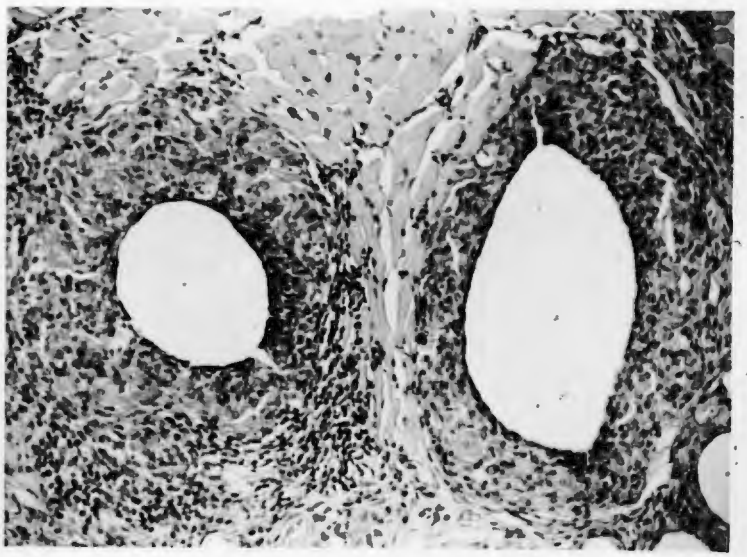


Figure 3. Cellular reaction surrounding oil droplets of adjuvant vaccine. Fourteen days after inoculation. (X 120)

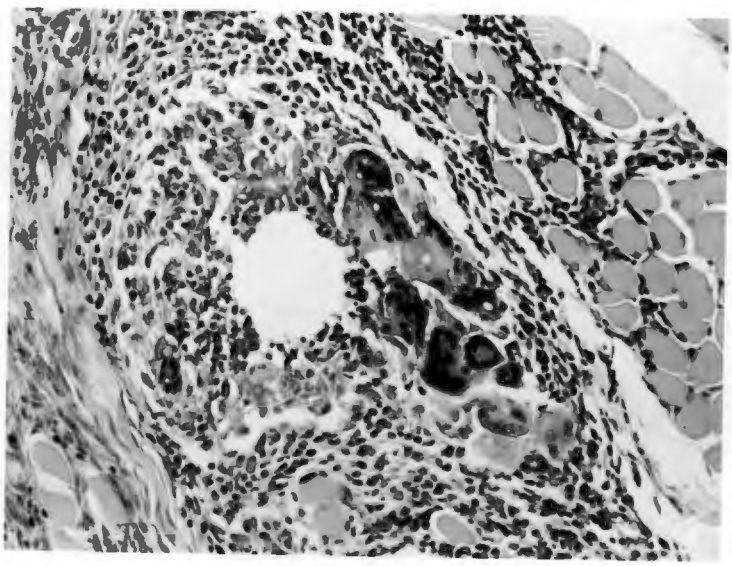


Figure 4. Foreign-body giant cells near oil droplet. Fourteen days after inoculation. (X 240)

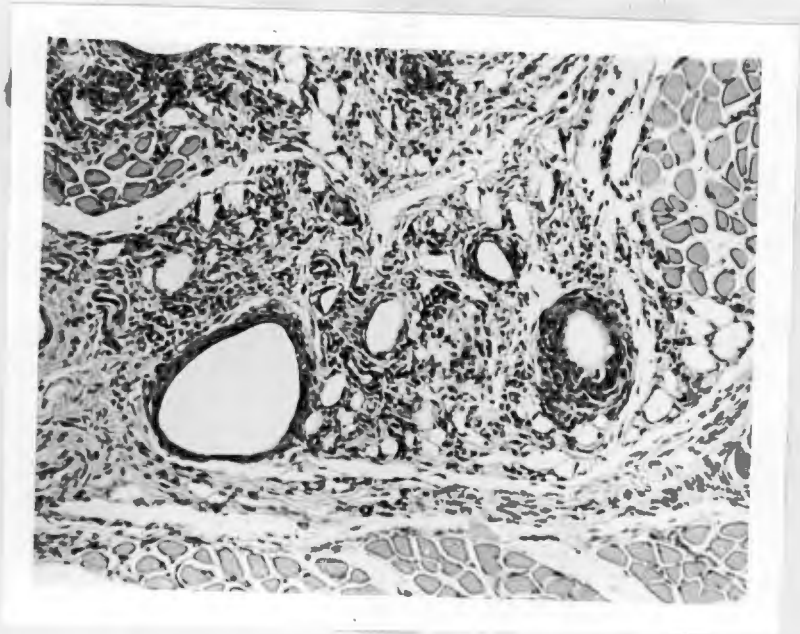


Figure 5. 52 days after the inoculation of adjuvant vaccine intramuscularly. (X 120)

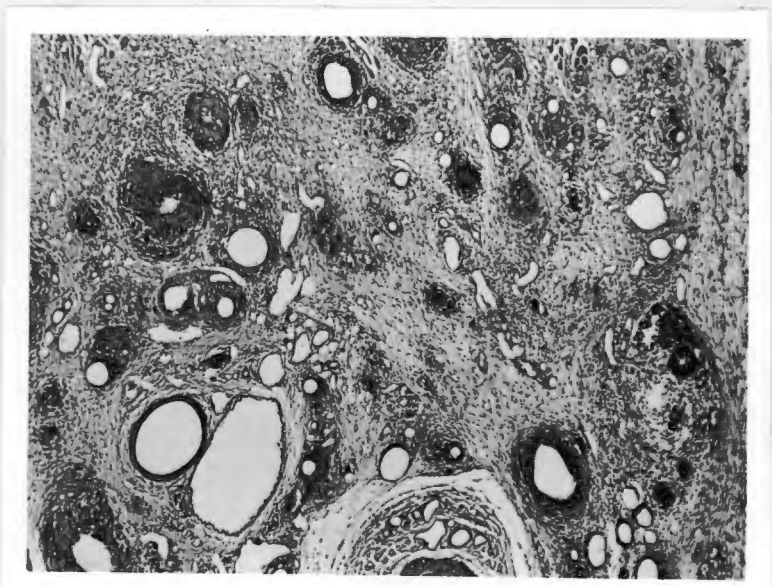


Figure 6. Sub-cutaneous area, 14 days after inoculation of adjuvant vaccine. (X 38)

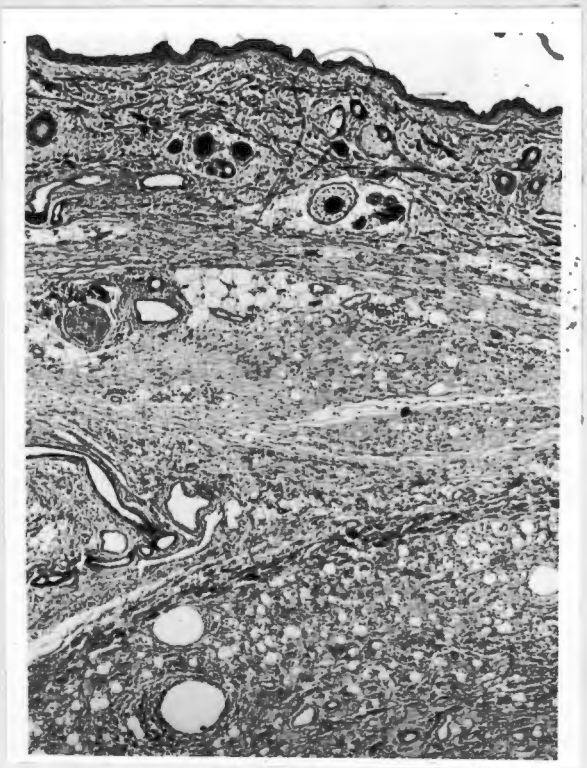


Figure 7. Sub-cutaneous area, 52 days after inoculation of adjuvant vaccine (x 30).

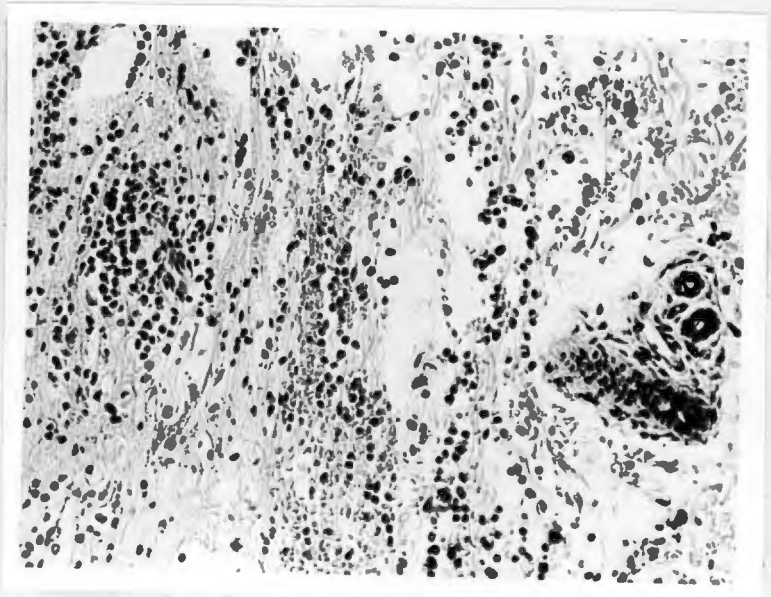


Figure 8. Sub-cutaneous area, 1 day after inoculation with aqueous vaccine (x 240).

were a little swollen compared with those on the uninoculated side and reactive germinal centres were seen with any characteristic changes.

Aqueous Vaccine

Aqueous vaccine produced a very different and much more transient type of response. One day after subcutaneous inoculation of aqueous vaccine the tissues were swollen and oedemations and profusely infiltrated with polymorphs (Figure 8). This reaction also was visible between the underlying muscle bundles.

SECTION C

INFLUENZA ANTIBODY RESPONSES

The antibody response to the doses of vaccine given was determined by tests on sera taken from the volunteers as has been indicated above. The work was carried out in three main sections.

Trial 1

Group A

In this section the sera taken before and 21 or 28 days after the two inoculations were examined. Thus, there were four sera each from those volunteers who had taken part in both parts of the first trial and two sera from those who only attended one part of the trial. The period of surveillance after the inoculation in the first part of the trial was shorter because at this time only 3 weeks were available before the students departed on vacation.

All the sera were examined by the Haemagglutination Inhibition (H.I.) test and a proportion of the sera were also tested for neutralizing antibodies to the same viruses as were used in the H.I. tests.

Group B

Here the sera that were taken 9-10 months after the second inoculation were tested. The previous two sera of

each volunteer were tested at the same time to establish a base line.

In addition to the two main influenza virus strains present in the vaccine many of these sera were tested by H.I. against a spectrum of different influenza viruses.

(NOTE: Groups A and B of Trial 1 allude to the way in which the antibody studies were divided and are different to the Parts I and II of the Trial itself.)

Trial 2

From the volunteers in the second Trial sera taken at the time of inoculation and one month and three months after inoculation were available. These sera were tested by H.I. against a number of different strains of influenza virus.

Materials and Methods

Sera

The bloods, when taken, were labelled with the name of the volunteer and the date. However, the separated sera were labelled, in addition, with the volunteer's number (between 1 and 433 in the first trial and 501 and 629 in the second - as the system was only partly consecutive) and a letter signifying the time of bleeding. These code letters were as follows and the sera were tested in the

groups indicated:-

Trial 1

Part I			
Pre-inoculation specimen	November, 1962	W	Group A
21 days after inoculation	December, 1962	X	
Part II			
Pre-inoculation specimen	January, 1963	Y	Group B
28 days after inoculation	February, 1963	Z	
9-10 months after inoculation	October-November, 1963	B	

Trial 2

Pre-inoculation specimen	November, 1963	F	Tested together
One month after inoculation	December, 1963	G	
Three months after inoculation	February, 1964	H	

As the sera of Trial 1, Group A and of Trial 2 were also tested for blood group antibodies the letters were chosen to avoid confusion with those commonly used in haematological practice. Unfortunately, this caused the letter "W" to refer to the first serum of Trial 1 as well as to the Placebo vaccine.

Hameagglutination - Inhibition Test

The technique of the test and of the treatment of sera to remove non-specific inhibitors is described in Appendix 2.

Ordering of the test

1. In each section of the work all sera from a person were tested against any one virus at the same time so that any pattern of change of titre would not be affected by day-to-day variations in the test.

2. Specific immune sera prepared in animals (Appendix 3) were included in all tests but titres were not adjusted from the results obtained from these controls. If the titres of the standard sera showed that the test done on a day was unsatisfactory the day's work was repeated.

3. While testing for antibodies to A/Singapore/1/57 and B/England/939/59 in Trial 1, Groups A and B sera were tested against both viruses on the same day. In Trial 1, Group B other antigens were tested against only a limited number of sera and here, as in Trial 2, it was possible to test all the sera involved against any one antigen on the same day.

4. Sera were tested in numerical order so that during the test there was no indication as to what vaccines had been administered to the volunteer concerned.

Vaccine groups

In Trial 1 four different inocula were given, two in November, 1962, and two in January, 1963. As a result the volunteers fell into different groups by virtue of the vaccines received. The inocula used were:-

November, 1962	1/	Placebo	0.5 ml.	W.
	2/	Aqueous vaccine	0.5 ml.	R'
January, 1963	1/	Adjuvant vaccine	0.25 ml.	O
	2/	Aqueous vaccine	1.0 ml.	R

(The aqueous vaccines were identical but R' signifies the 0.5 ml. dose as distinct from the 1.0 ml. dose which is designated R.)

Therefore, the following vaccine groups were possible:-

W - received only placebo

R' - " " 0.5 ml. of aqueous vaccine

WO - Placebo in Part I followed by adjuvant vaccine in Part II

WR - Placebo in Part I followed by aqueous vaccine in Part II

R'O - 0.5 ml. aqueous vaccine followed by adjuvant vaccine

R'R - " " " " " " 1.0 ml. aqueous vaccine

Numbers

The numbers of sets of sera examined in any vaccine group is not necessarily identical with the numbers of volunteers as shown in the section on vaccine reactions. This is because the titres of sera in the first trial were only included in the results when specimens before and after vaccine were available for comparison and when there was no doubt as to the vaccine received. A few sera were lost in laboratory accidents, e.g., tubes breaking in the

centrifuge. In certain cases sera became short after repeated tests of various types and thus could not be tested against all the antigens.

Presentation of results

Results are generally presented in the form of histograms showing the distribution of antibody titres and in graphs made up comparing the Median titres of sera. The Geometric Mean Titre (G.M.T.) is more frequently used and is both more sensitive and provides a better representation (Hirst et al., 1942 b). However, the Median Titre was used in the present work because, with some antigens, the number of titres found to be less than the lowest level of test would have produced a G.M.T. less than the level of test and hence quite invalid.

The histograms also show the median titre of each batch of sera tested. Where this could not be shown for reasons of space the value in each case is less than 10. With each histogram a scale indicates the numbers of sera. However, with most of the influenza B antigens there were so many sera with titres of less than 10 that, where necessary, the width of this column had to be doubled and, therefore, in these double width columns the numbers of sera are twice that indicated by the scale.

In the graphs the points depicting the median titres are joined by lines. These lines are included purely for

demonstration purposes. They do not attempt to indicate the median titres at times between the samples.

Trial 1

Part I

Results

The titres of sera taken before and 21 days after the administration of W and R' vaccines are shown in Figures 9, 10 and 11. In these and the following diagrams titres are expressed as the reciprocal of the serum dilution. Results are grouped in two-fold steps so that columns marked, say 10, include titres from 10 to 19. For the median values the interpolated titres are used. From those receiving placebo (W) 181 pairs of sera were tested and 190 pairs from volunteers injected with R'.

It is clear from the figures that whereas the placebo has not been associated with a general rise in titre, vaccine R' has caused a marked rise in titre. However, there is a sharp quantitative difference between antibody to A/Singapore/1/57 and B/England/939/59. The titres to influenza B are generally at a lower level and a very large proportion of sera have an initial level of less than 1:10. In addition, of the 101 volunteers receiving R' who had pre-inoculation H.I. titres to B/England/939/59 of less than 1:10, 25 did not respond to produce antibody detectable at this level. The proportion of "non-convertors" is the same with the influenza A strain but the numbers are vastly different - 2 out of 8.

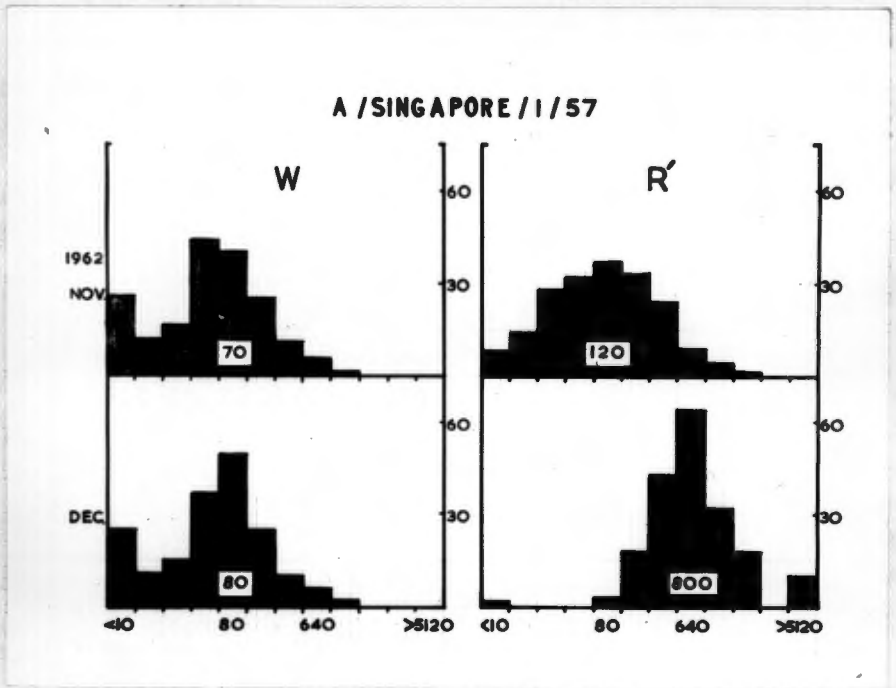


Figure 9. Histogram of antibody response in Trial 1, Part I.

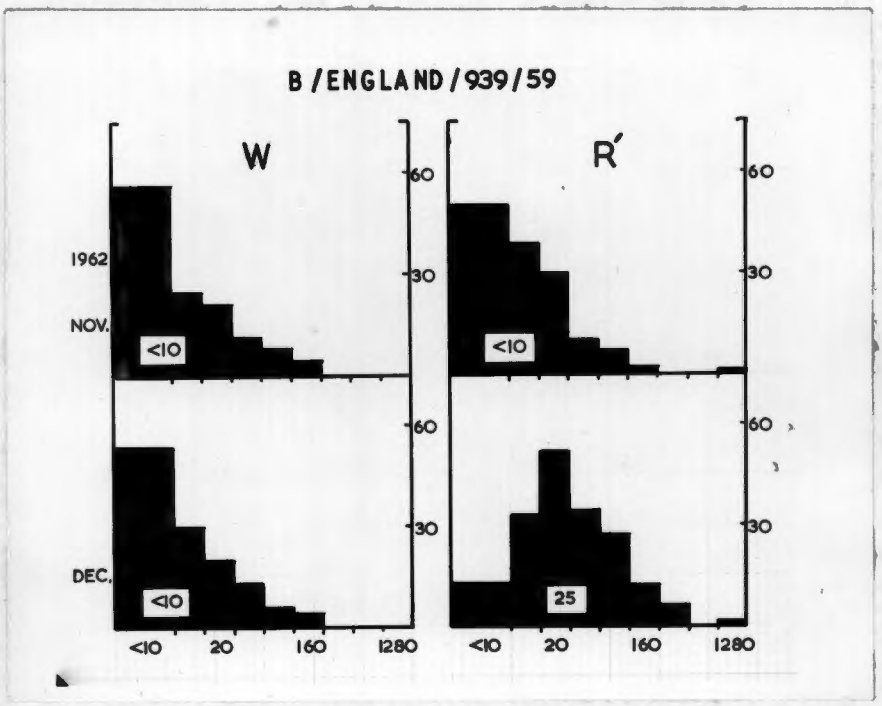


Figure 10. Antibody response in Trial 1, Part I.

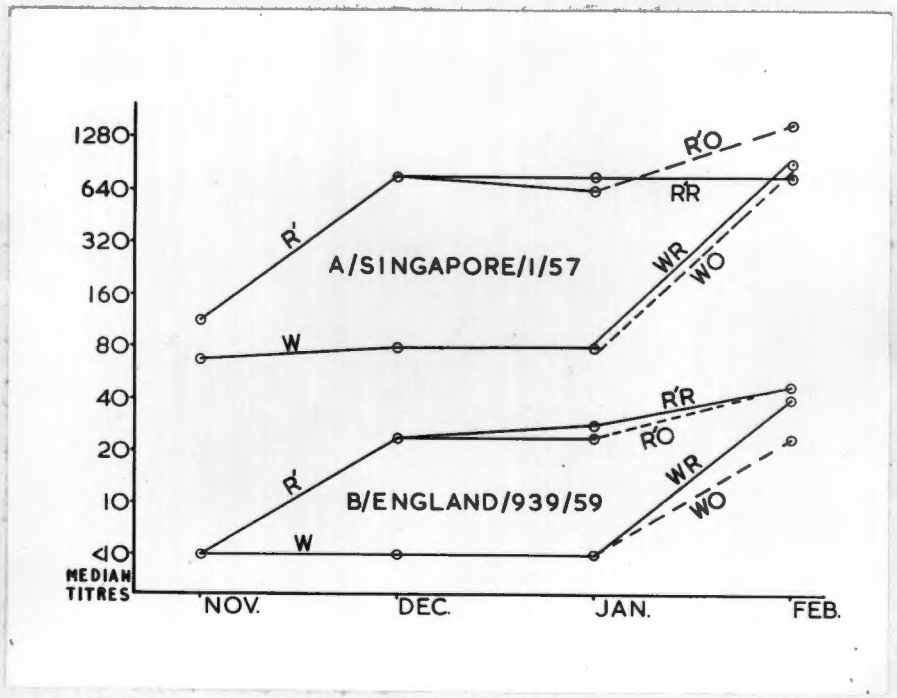


Figure 11. Graph of median titres during Trial 1. Broken lines indicate response after adjuvant vaccine.

Part II

From this part of the trial 158 pairs of sera were available from those who had received adjuvant and 183 from those who were given aqueous vaccine in the second part. They were divided into "vaccine groups" as follows:-

WO	WR	R'O	R'R
75	88	83	95

The three volunteers who only joined the trial at this stage were counted as having received placebo (W).

Results

Figure 11 shows that in the intervening month there was no great change in antibody levels. This figure and Figures 12 and 14 show that the vaccines both produced a clear antibody response in those who previously had placebo. The adjuvanted vaccine, containing but one-fifth the amount of antigen present in 1.0 ml. of aqueous vaccine, R, has produced comparable titres to A/Singapore/1/57 ($0.3 > P > 0.2$) but with influenza B the titres are distributed at a significantly lower level ($0.025 > P > 0.01$). In both cases, WO and WR, the sera before inoculation were comparable.

The median titres that result from this inoculation are not very sharply different from those conferred by R' which had only one-half the antigen given with R'.

Regarding the "conversion" of those with no antibody

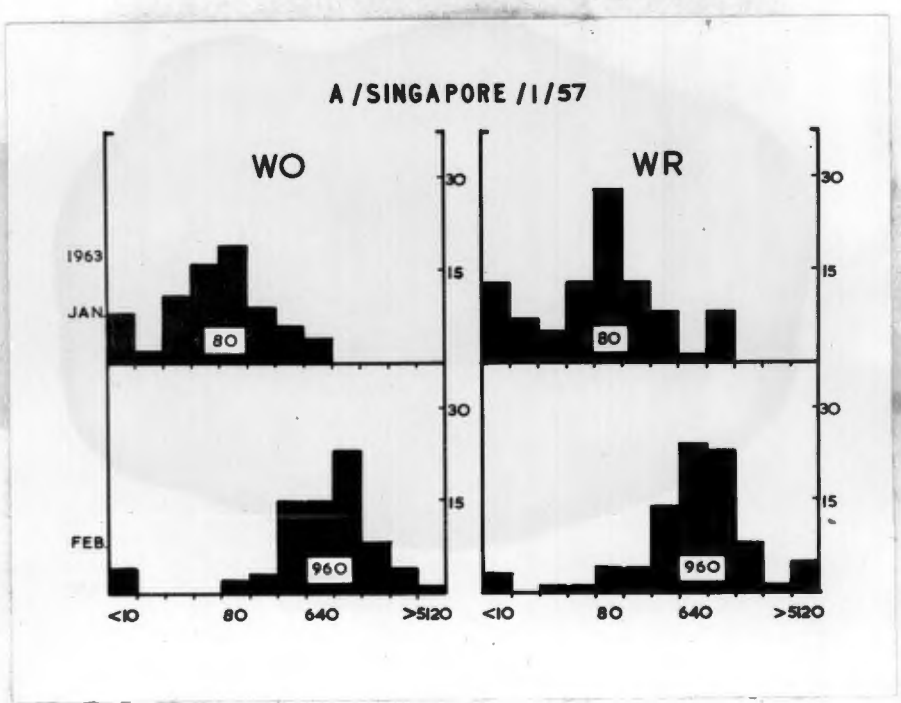


Figure 12. Antibody response in Trial 1, Part II, following an initial inoculation of placebo.

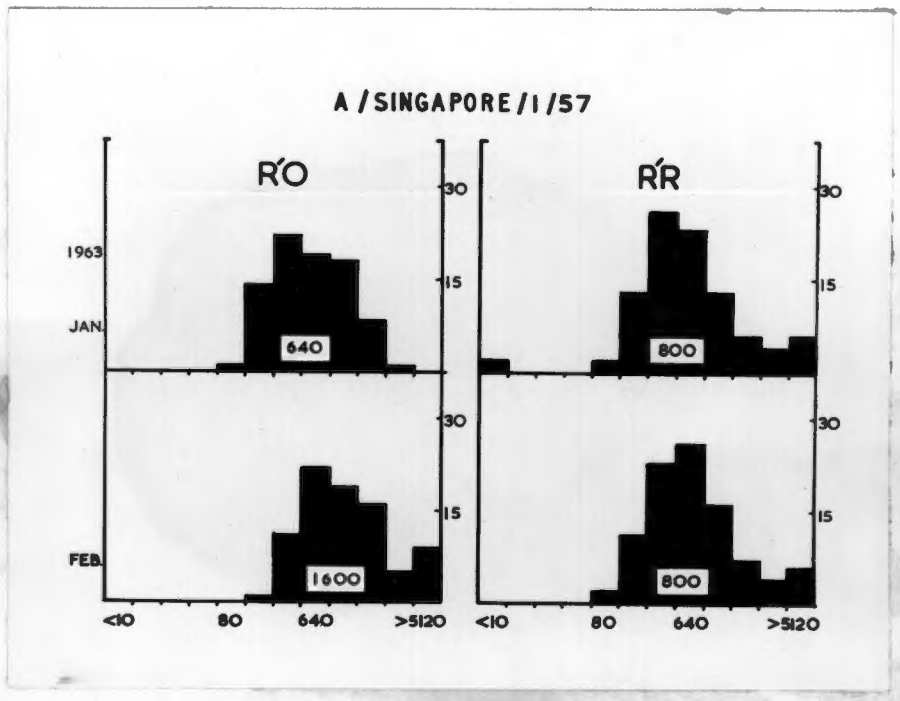


Figure 13. Antibody response in Trial 1, Part II, following an initial inoculation of 0.5 ml. of aqueous vaccine.

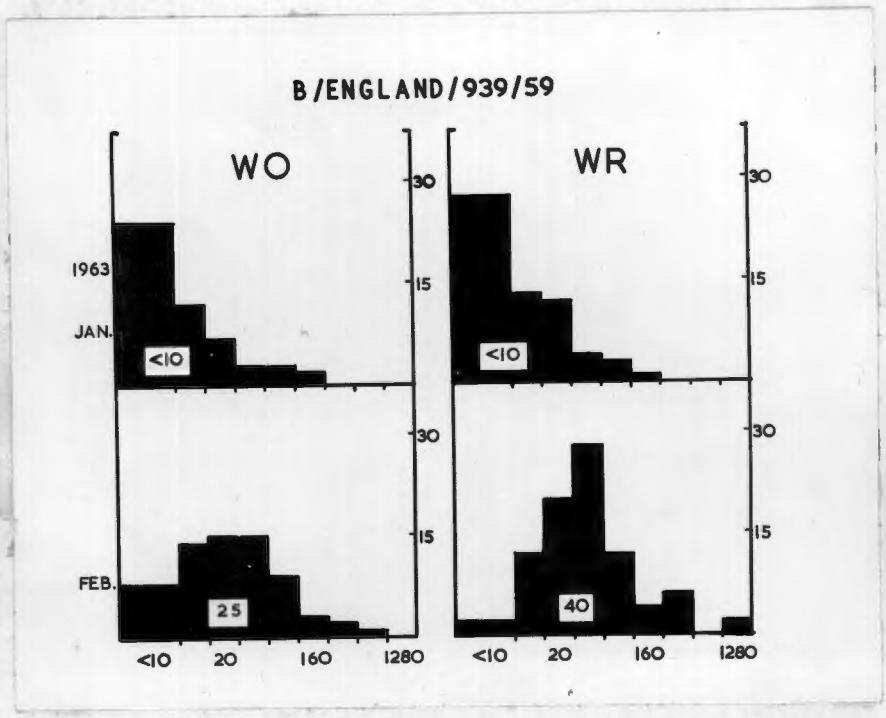


Figure 14. Antibody response in Trial 1, Part II, following an initial inoculation of placebo.

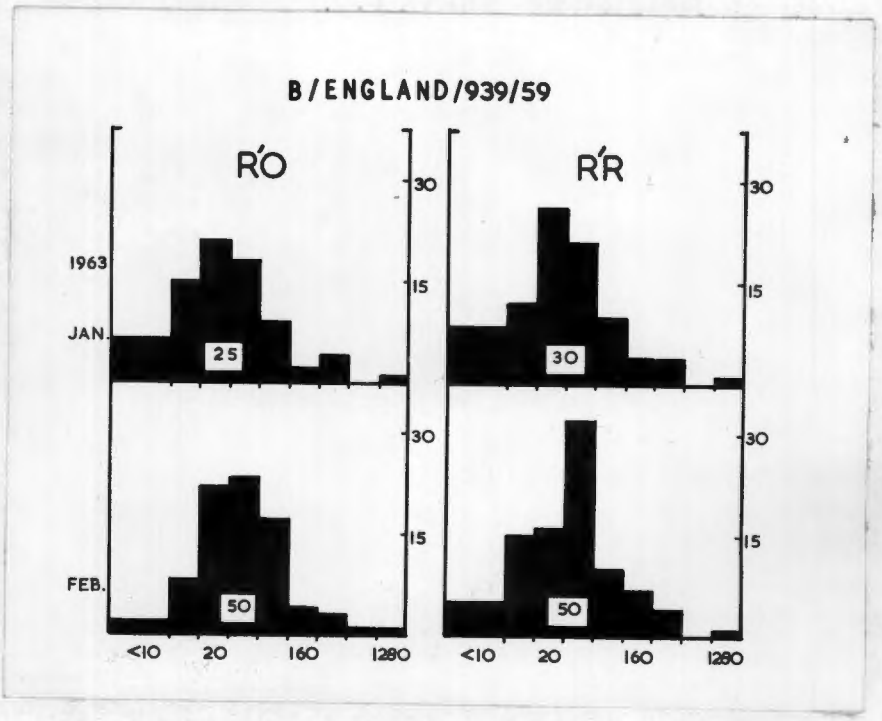


Figure 15. Antibody response in Trial 1, Part II, following 0.5 ml. of aqueous vaccine initially.

to the influenza B strain detectable at 1:10 before inoculation, one-third (16 of 48) of those receiving adjuvant had not shown antibody by 28 days after inoculation but only one-fourteenth (4 of 55) of those receiving aqueous vaccine had not converted. This difference is significant ($0.001 > P > 0.0005$). To a smaller extent the same is true with influenza A where, in response to "O" vaccine 4 of 8 converted (50%) but 10 of 13 (77%) showed antibody with "R" vaccine ($P = 0.345$).

When the group receiving R' vaccine in the first part is considered in Figures 11, 13 and 15 it is clear that no clear secondary response, in the usually accepted sense, has occurred. The degree of rise of antibody level is small and in the case of influenza B there is no difference between the types of vaccine used. However, with influenza A a higher median titre has been produced by the adjuvant vaccine and the significance of the degree of difference is confirmed when the distribution of the titres of those in the two vaccine groups are compared ($0.005 > P > 0.001$). In addition, the apparent efficacy of vaccines in causing "conversion" is reversed, with "O" producing detectable antibody in 9 of 13 (70%) and "R", 7 of 17 (41%) but the difference is not significant. ($0.2 > P > 0.1$).

After the titrations of Group A had been completed it was clear that the titres of antibody to B/England/939/59

were abnormally low in those volunteers whose sera had been tested in the first weeks. It was shown that this was due to a technical error when the same sera were tested during Group B and the titres compared. As a result all the titrations of B/England/939/59 in Group A were repeated and this time the titres were of a similar order throughout and corresponded with those obtained in Group B titrations. The results shown refer to the repeated titrations. A number of attempts to discover the cause of this discrepancy, involving the use of different batches of antigen to test for avidity, were unsuccessful.

Trial 1

Group B

In this section of the work the sera obtained in October/November, 1963 (i.e. "B" sera), from those who had taken part in the second half of Trial 1 and had received either adjuvant vaccine or 1.0 ml. of aqueous vaccine were compared with the Y and Z sera of the same volunteers in parallel titrations.

301 sera were obtained from the 341 volunteers eligible. It was not possible to trace the rest. Some had left the university and others were working in different hospitals. Those volunteers who were bled were distributed among the vaccine groups as follows:-

	WO	WR	R'O	R'R
Number	68	74	79	79
% of whole group	92.0	84	95	83

Relatively more of those who had received adjuvant vaccine were bled. Although this distribution falls outside the chance level ($0.05 > P > 0.025$) it is difficult to know which factors could be blamed for this. To attribute it, say, to differences in reaction between the vaccines received months earlier would be presumptuous.

All these sera were tested for antibodies to A/Singapore/1/57 and B/England/939/59. The sera of vaccine groups WO and WR were, in addition, tested against:-

A/Netherlands/65/63

A/FM$_1$/47

B/England/10/54

B/Taiwan/4/62

B/Lee/40

Parainfluenza 1 (Sendai).

These additional tests were done to examine for the greater antigenic spread attributed to adjuvant vaccines, to test whether there was any "recall" of antibodies to past strains of influenza no longer prevalent and to examine whether the effect of adjuvant vaccine was related to any non-specific effect on antibody production

as might be suggested by the work of Neeper and Seastone (1963). One of the volunteers in vaccine group WO had to be excluded from the tests against additional antigens owing to the depletion of his "Z" serum by haematological tests.

As the decision to test against B/Lee/40 was stimulated by the results of later work when the treated sera were exhausted and there was only a limited amount of cholera filtrate available it was necessary to remove inhibitors from some of the sera by the trypsin-periodate method (Appendix 2) and this required special representation.

Part of the sera falling into vaccine groups R'O and R'R were tested against A/PR-8/34 and A/Swine (Shope 15) in order to test the effect of the vaccine on antibody to these old antigens. With some of these tests pigeon cells were employed in parallel with chicken red cells to exploit the greater sensitivity (Berlin, McQueen, Minuse and Davenport, 1963) of the former.

It was not thought worth while to test the sera against the strain A/England/1/61, although it was incorporated in the vaccine, both because it is so closely related to A/Singapore/1/57 and because this strain seems to have been an off-shoot from the main line of evolution of the Asian (A_2) set of viruses and has not occurred elsewhere since (Pereira H.G., 1963).

Results

The results from tests with A/Singapore/1/57 are shown in Figures 16, 17 and 18 and those with B/England/939/59 in Figures 19, 20 and 21. In the graphs, broken lines connect the median titres in the vaccine groups receiving adjuvant vaccine.

With influenza A the titres achieved with adjuvant vaccine are clearly superior at the 9-10 month mark, both in the group which had received placebo and that which had had a preliminary dose of vaccine, R'. This superiority is reflected in the median titres and the distribution of the numbers of sera among the levels of antibody were significantly different (for WO/WR $0.01 > P > 0.005$, and for R'O/R'R, $P < 0.0005$).

Regarding antibodies to influenza B, the superior duration of effect of adjuvant vaccine is once more apparent although the difference between the vaccine groups WO and WR shown in the distribution among titres is within the limits of chance ($0.2 > P > 0.1$). The graphs also show that the influenza B antibody response to adjuvant vaccine is delayed, compared with that of influenza A.

With both viruses the serum titres of a number of volunteers receiving aqueous vaccine had fallen to levels of less than 1:10 by 9-10 months. With adjuvant vaccine

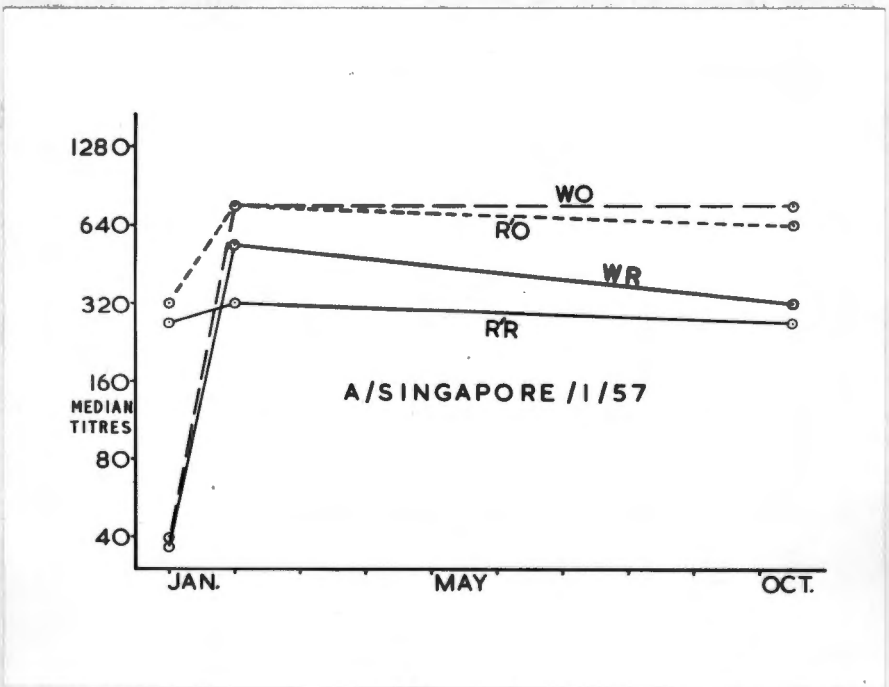


Figure 16. Graph showing persistence of antibody titres after 9-10 months.

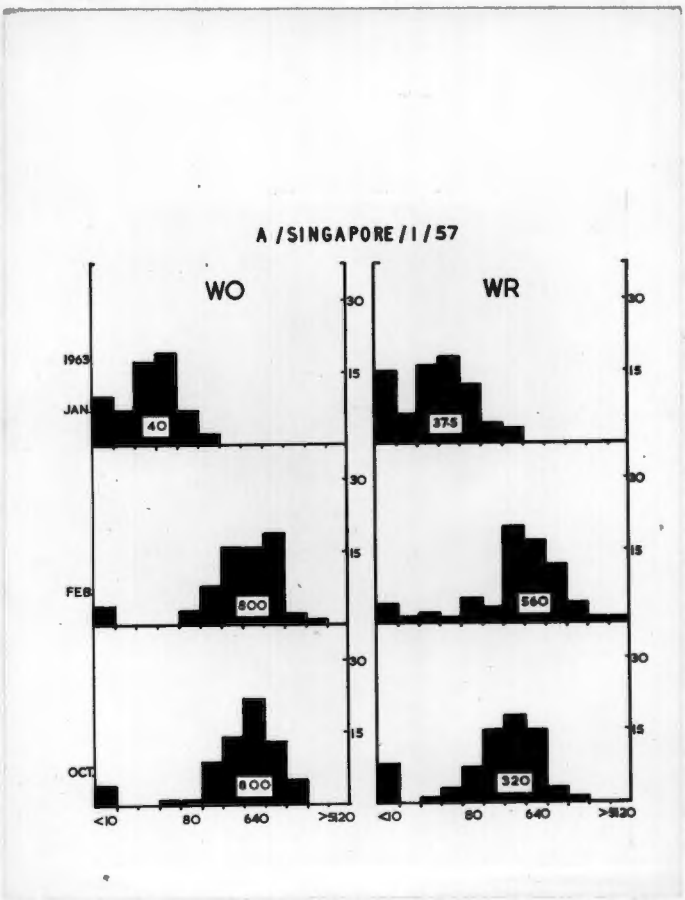


Figure 17. The persistence of antibody titres following a single inoculation of vaccine. Trial 1.

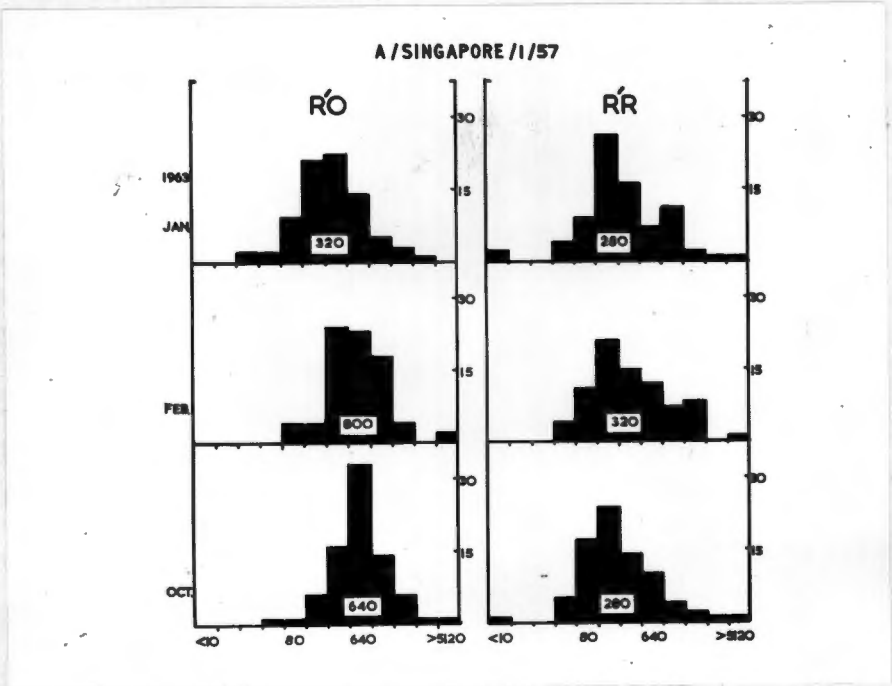


Figure 18. . The persistence of antibody titres following two inoculations of vaccine. Trial 1.

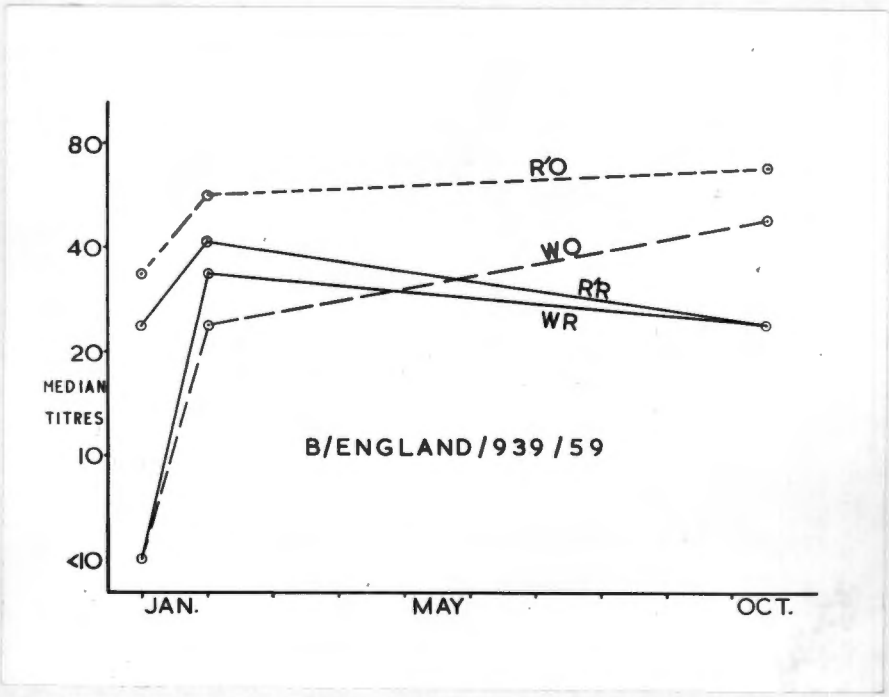


Figure 19. Graph showing persistence of antibodies after 9-10 months. Trial 1.

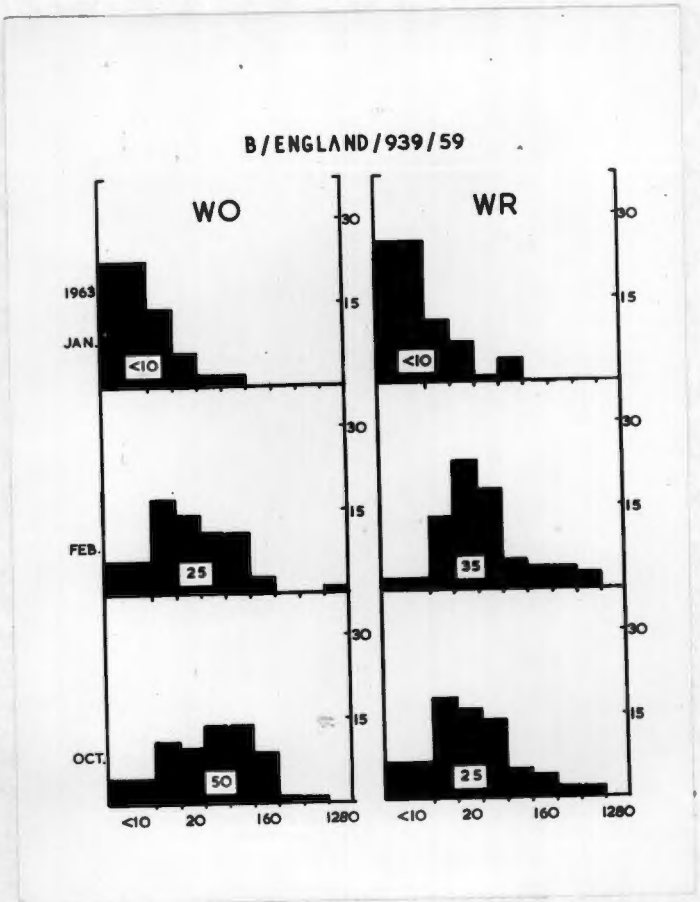


Figure 20. Persistence of antibody levels after one dose of vaccine. Trial 1.

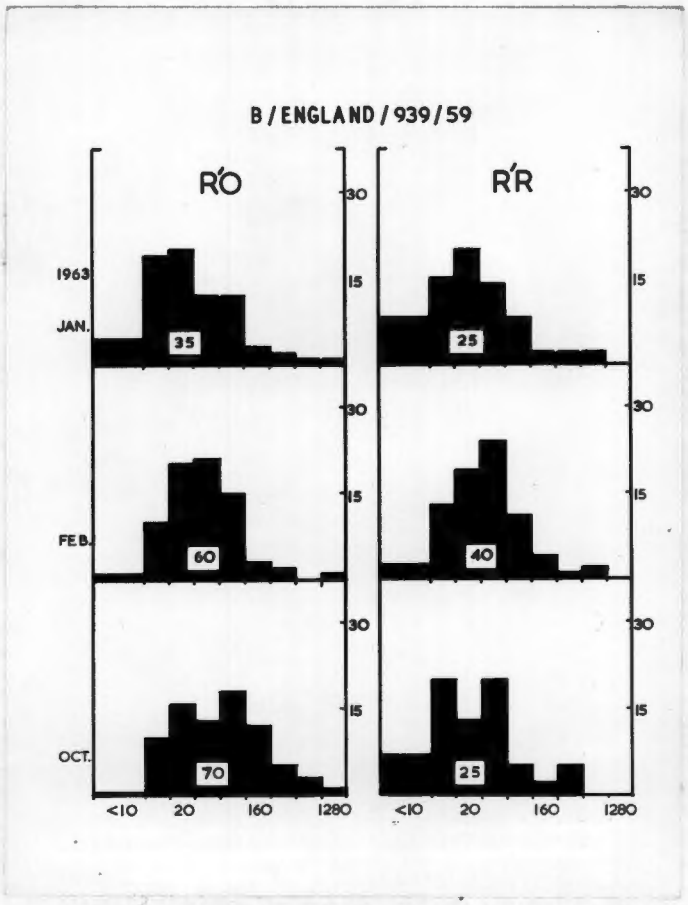


Figure 21. Persistence of antibodies following two doses of vaccine. Trial 1.

and antibody to influenza B this tendency is actually reversed showing that some people "converted" more than four weeks after receiving vaccine and their antibody remained at detectable levels for the remaining months.

Titrations of other strains of influenza A

From Figures 22 and 23 it is clear that titres to the Asian variant A/Netherlands/65/63 were closely parallel to these of the vaccine strain and Asian prototype, A/Singapore/1/57. This occurs with both vaccines. However, Figures 22 and 24 show that neither vaccine has any effect upon antibodies to the A_1 family prototype, FM_1.

The results of titration of the R'O and R'R vaccine groups with A/PR-8 and A/Swine antigens are shown in Figure 25. In group R'O the sera of 31 volunteers were tested against A/PR-8 and 28 against A/Swine and in group R'R the numbers were 35 and 31 respectively. All the various classes of the students were represented in all groups but no specific randomising procedure was adopted.

There was no indication that two inoculations of the vaccine recalled antibodies from a significant number of people nor is there evidence that the second inoculation, of either vaccine, caused more than a minimal increase of antibody in those who already possessed it. The three people who showed antibody to A/Swine were born in 1934, 1936 and 1941. Two of these were African students. Only

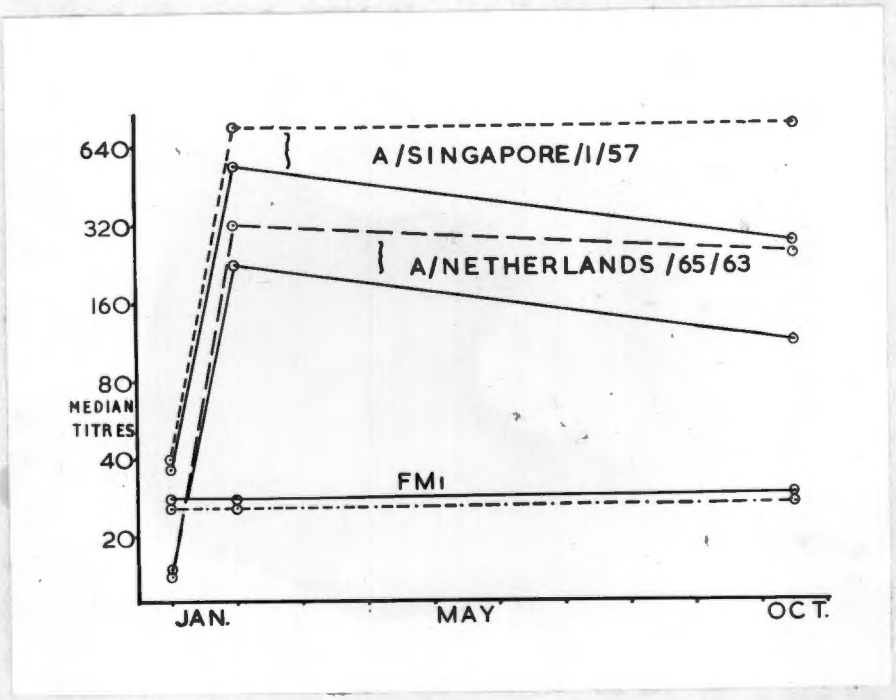


Figure 22. Graph showing effect of vaccines on antibody to various influenza A antigens. Trial 1.

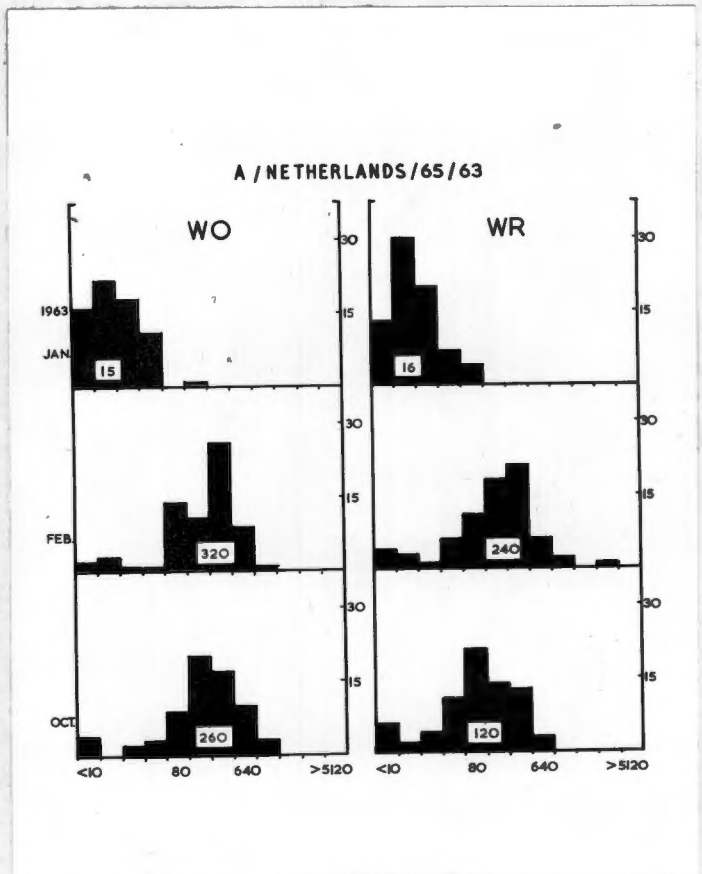


Figure 23. Distribution of titres of antibody to A/Netherlands/65/63. Trial 1.

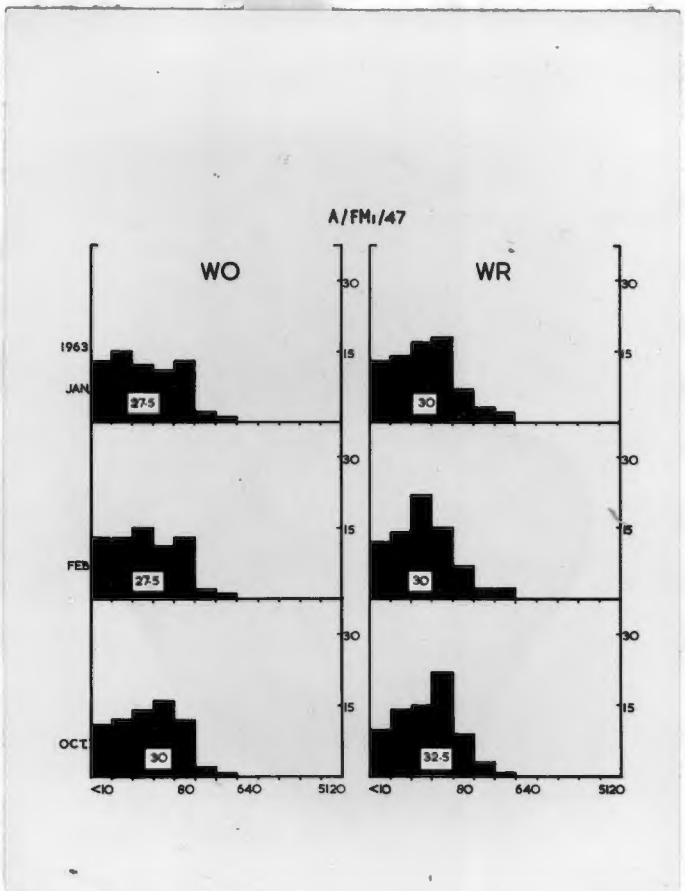


Figure 24. Distribution of titres of antibody to A/FM$_1$. Trial 1.

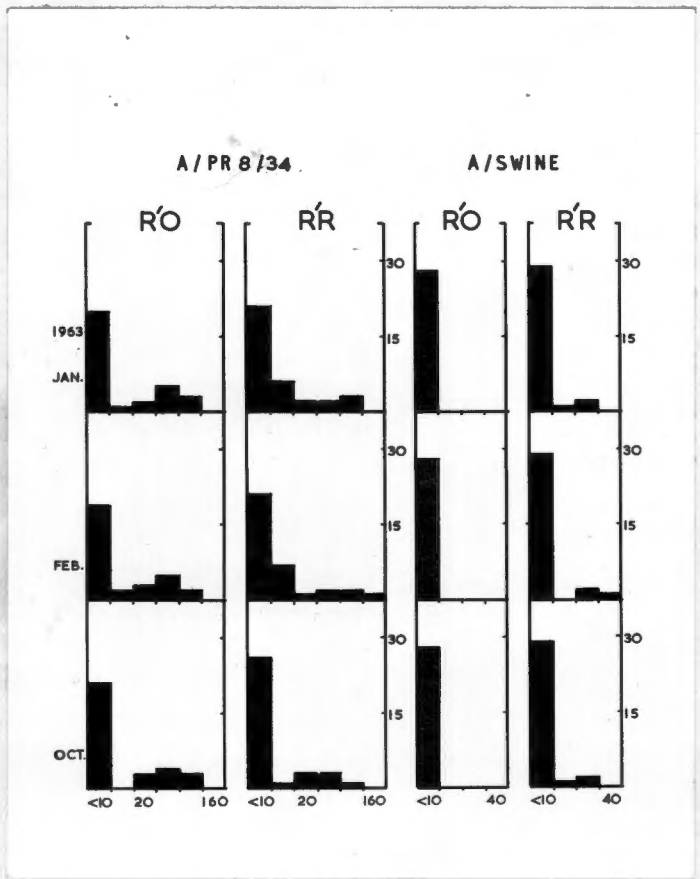


Figure 25. Distribution of titres of antibody to A/PR-8 and A/Swine strains. In all cases the median titre was less than 10.

one had antibody to A/PR-8 as well.

Much more antigen was needed to prepare 8 H.A. unit antigen for pigeon cells than for chicken cells. Pigeon cells, and the special antigen for it, were used with about half the sera titrated with each antigen.

The sera of 60 volunteers were tested with pigeon cells, half against A/PR-8 and half against A/Swine. In those cases where antibody had been detected using chicken red cells rather higher titres were obtained with pigeon cells but there were exceptions to this. The use of pigeon cells failed to reveal any reaction to the vaccines which the test with chicken cells did not.

Titration of antibody to other strains of influenza B virus.

With the influenza B strains a rather different situation was observed. From Figure 26 and the histograms, Figure 27, it appears that the vaccines, containing B/England/939/59, caused a rise in antibodies to B/England/10/54 but that with adjuvant vaccine this rise seemed to be more transient than that to the homologous strain. In fact, the antibody to B/England/10/54 induced by both vaccines behaved like that to B/England/939/59 produced by aqueous vaccine. However, antibodies to B/Taiwan/4/62 and B/Lee/40 (Figures 28 and 29) were affected to a much smaller extent.

With B/Lee the antibody titres found in the sera inactivated by trypsin and periodate were slightly higher

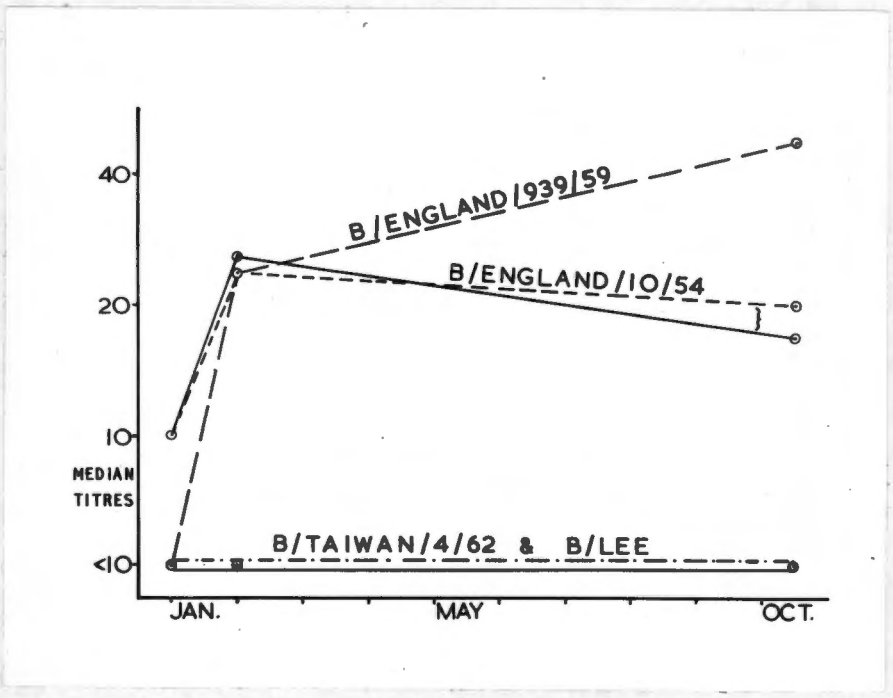


Figure 26. Graph showing response of antibody to different strains of influenza B virus. To simplify the graph that line relating to the B/England/939/59 antibody response to aqueous vaccine has been omitted. Trial 1.

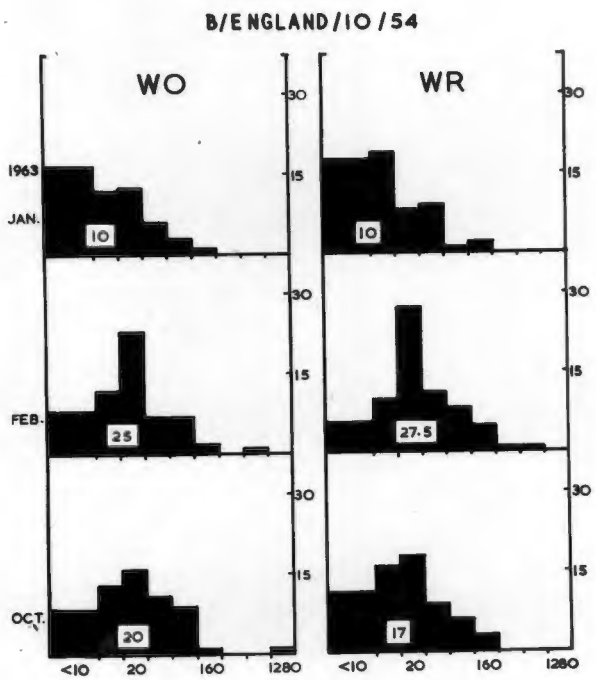


Figure 27. Histogram showing response of antibodies to B/England/10/54. Trial 1.

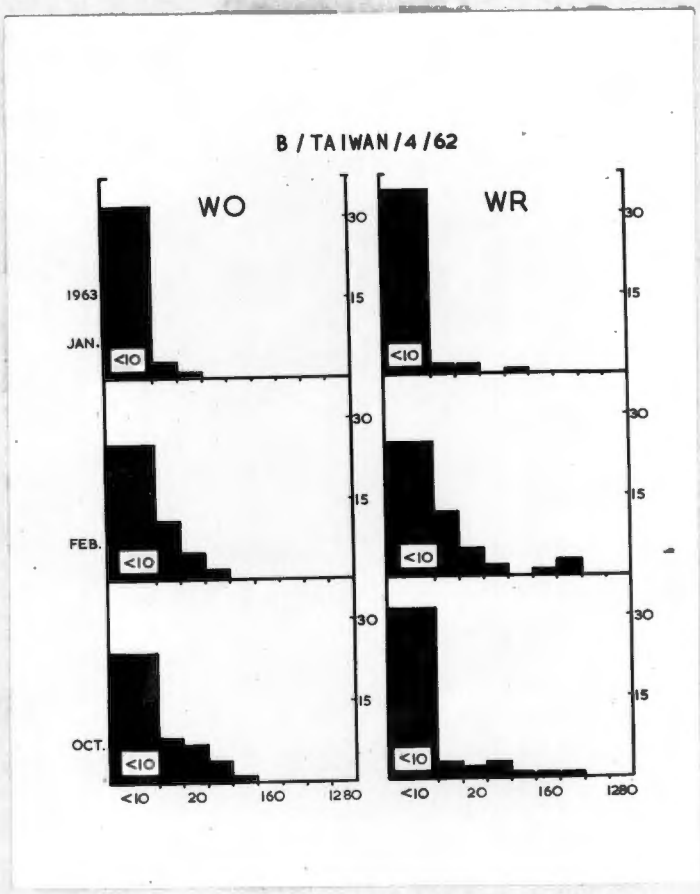


Figure 28. Histogram showing response of antibodies to B/Taiwan/4/62. Trial 1.

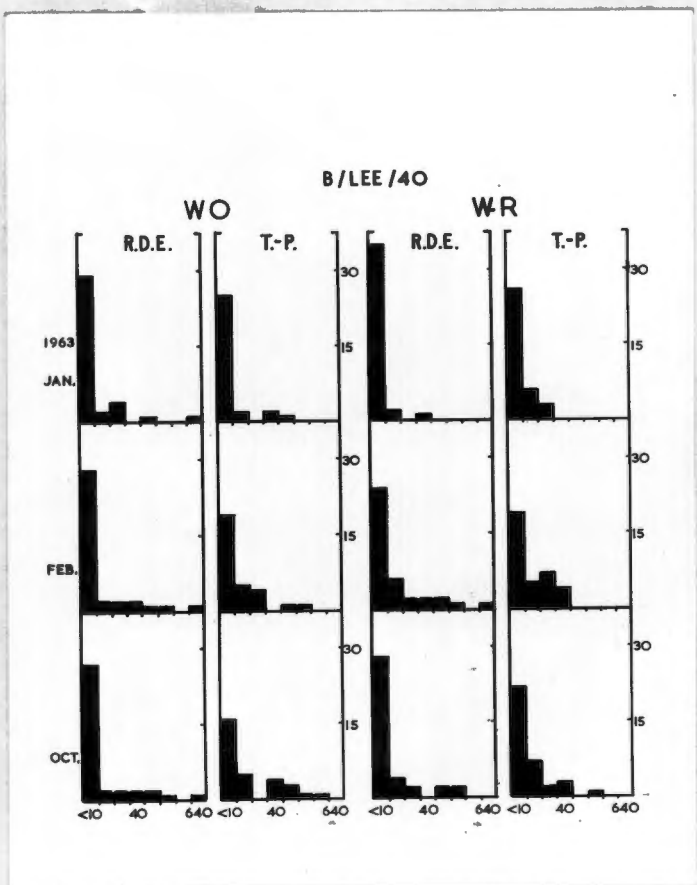


Figure 29. Histogram showing response of antibodies to B/Lee. The results from sera inactivated with Cholera filtrate and Trypsin-periodate are shown separately. In all cases the median titres were less than 10. Trial 1.

than those from sera inactivated with cholera filtrate. This unexpected result could possibly be related either to incomplete removal of inhibitors in the one case or to the destruction of antibody in the other. In selecting sera for one mode of treatment or the other an attempt was made to spread each equally among vaccine groups and student classes. However, no specific means of randomisation were employed. It was, therefore, not possible to combine the results although the same general tendency, of showing only a slight response to the vaccine, was the same for sera treated in either manner.

Para-influenza 1 (Sendai).

As shown in Figure 30, neither vaccine affected the antibody titres to Para-influenza 1 although, not unexpectedly, more than half of the volunteers had demonstrable antibody to this virus.

Trial 2

For the influenza antibody investigations of Trial 2, sera taken before inoculation and three months after inoculation were available from 97 volunteers but one volunteer was not bled at the session one month after vaccination. In addition, so little blood was obtained from one student three months after inoculation that there was insufficient material to titrate it against all the strains. However, the results of all available sera were

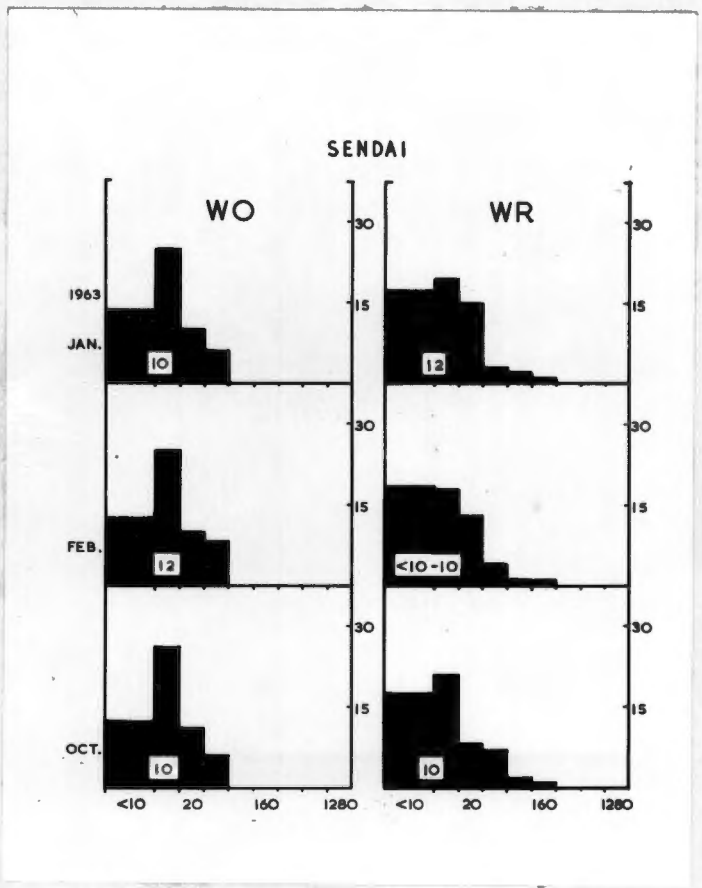


Figure 30. Histogram showing the effect of the vaccines on antibody to Para-influenza 1, Sendai. Trial 1.

incorporated in the data throughout.

In the graphs of this trial broken lines do not signify a particular vaccine as all the volunteers received the same adjuvant vaccine. However, different patterns of lines were used for different strains of virus.

Results

The graphs, Figures 31 and 34 show that in response to adjuvant vaccine the antibody titres continued to rise after a month.

From Figures 31 and 32 it would seem that the relation between the two variants of the Asian set of viruses is even more apparent. The titrations against A/Netherlands/65/63 were twice repeated and gave the same result.

Although the titres of antibody to A/EM_1, shown in Figures 31 and 33, appear to be at a higher level than in Trial 1 - either because of the different population or because of an increased sensitivity of the test - the general result is unchanged. The vaccine had no appreciable effect upon the antibody to this virus.

The results obtained with strains of influenza B, shown in Figures 34, 35 and 36 differed fairly markedly from those in the first trial. With B/England/10/54 the fall in titre after an initial rise, demonstrated in the first trial, is not shown although the differences between the results of the two trials might only reflect

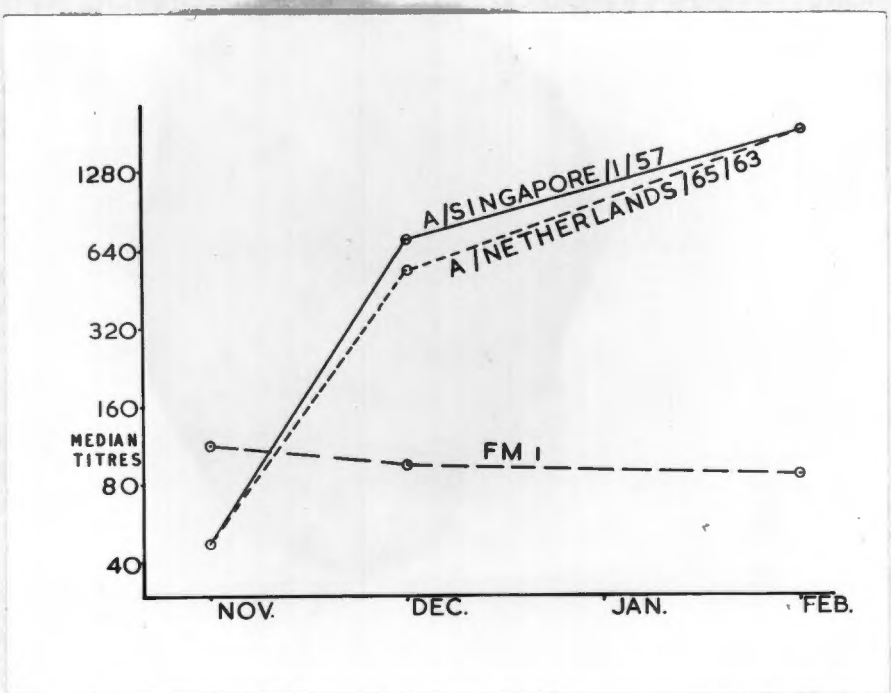


Figure 31. Graph showing response of antibody to various strains of influenza A virus. Trial 2.

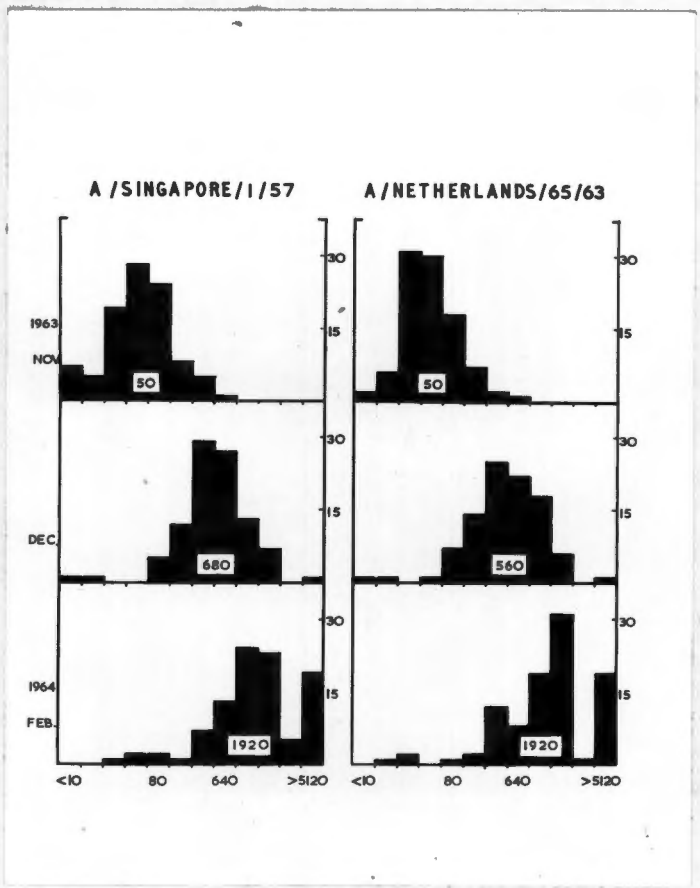


Figure 32. Histogram showing distribution of antibody titres to Asian strains of influenza A virus. Trial 2.

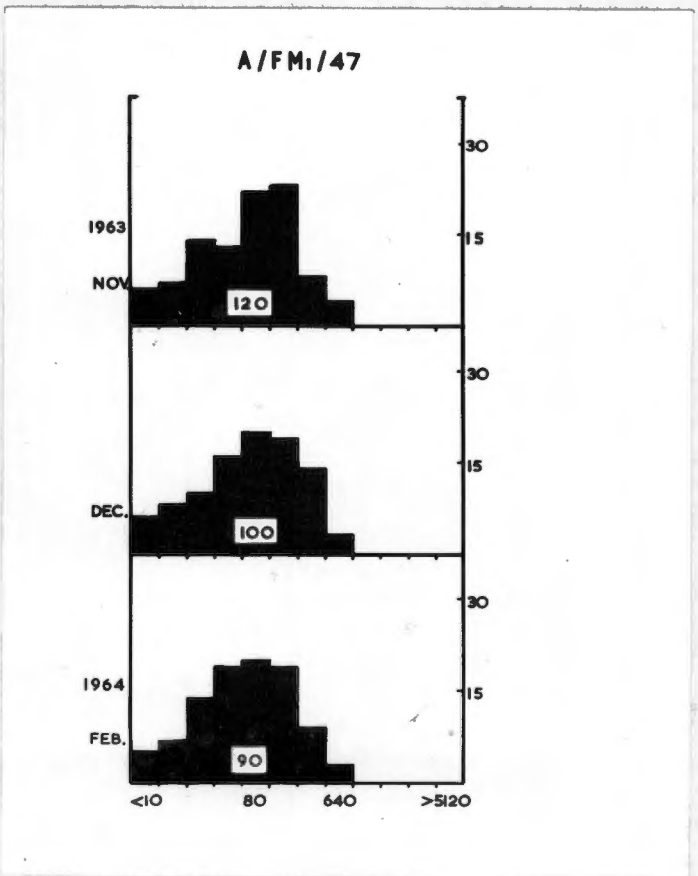


Figure 33. Histogram showing distribution of antibody titres to A/FM_1 strain. Trial 2.

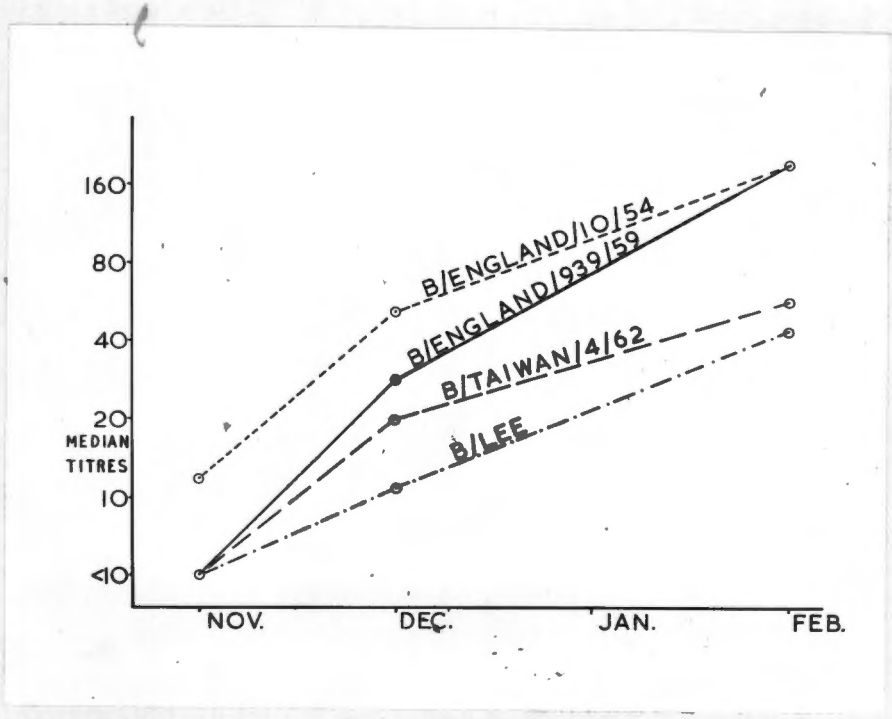


Figure 34. Graph of response of antibody to various strains of influenza B virus. Trial 2.

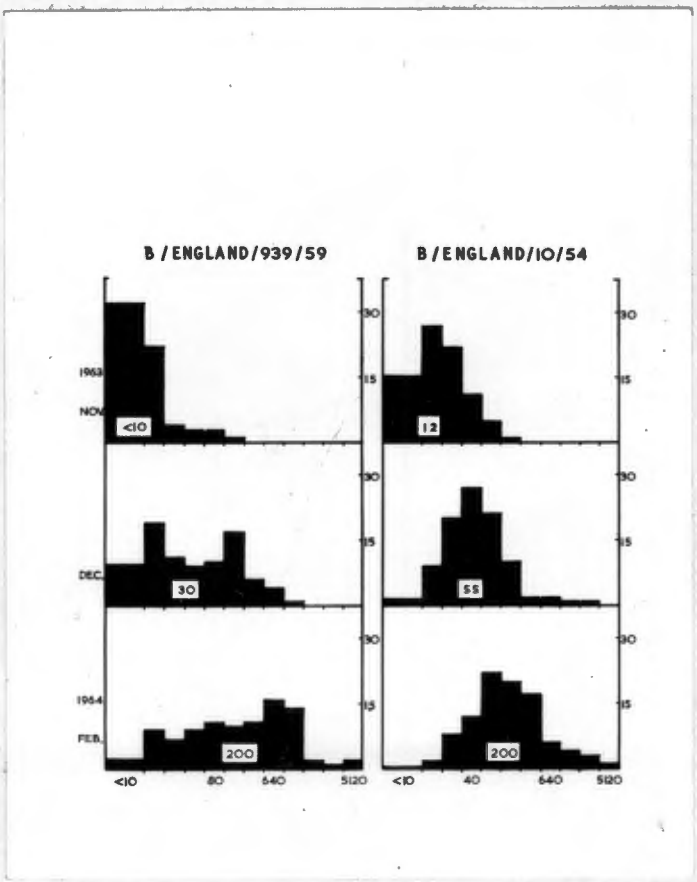


Figure 35. Histogram showing distribution of antibody titres to B/England/939/59 and B/England/10/54. Trial 2.

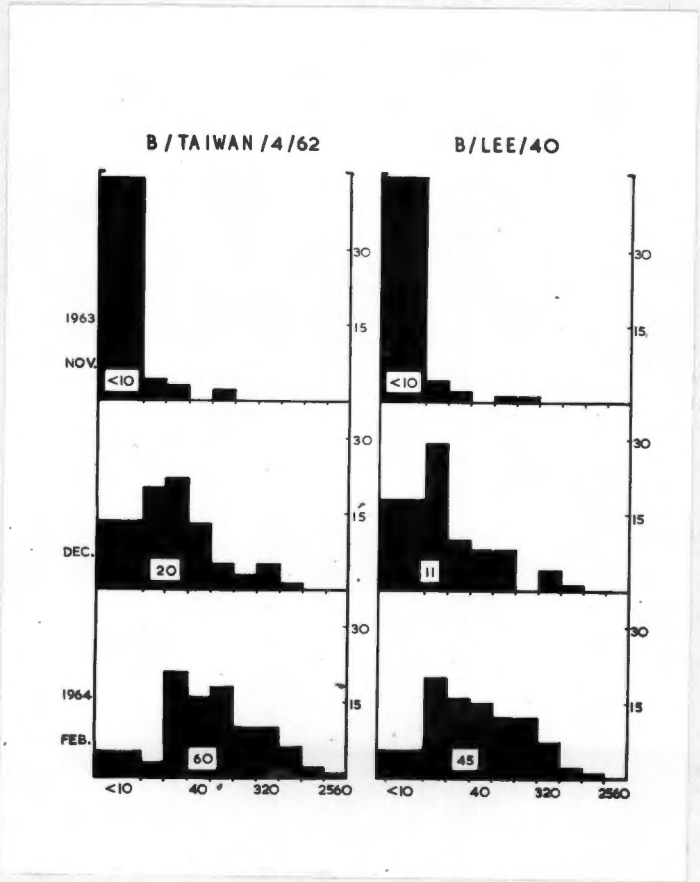


Figure 36. Histogram showing distribution of antibody titres to B/Taiwan/4/62 and B/Lee. Trial 2.

the difference in times of collection of the serum specimens. The titres to both B/England/939/59 and B/England/10/54 rose to greater levels than might have been suspected from the results obtained in the first trial.

Antibodies to B/Taiwan/4/62 and B/Lee/40 are both clearly affected by the vaccine, in contrast to Trial 1. As this is shown at one month also this difference is probably valid.

The vaccine used in Trial 2 contained a new component in B/Taiwan/4/62, which differed fairly markedly from the previously prevalent virus. It is possible that when a volunteer already had antibody to B/England/939/59 a vaccine containing both this antigen and the new B/Taiwan might stimulate the formation of antibodies to the old, to the exclusion of those to the new strain, B/Taiwan.

When the post-inoculation sera of those volunteers (24 in number) who had antibody to B/England/59 before inoculation and none (at a 1:10 level) to B/Taiwan were compared with the post-inoculation sera of those 64 volunteers who had antibody to neither virus before inoculation it was found that the median titres of B/Taiwan antibody were higher in the former group.

	Pre-inoculation antibody to B/England/939/59	
	Present	Absent
Median titre to B/Taiwan/4/62		
at 1 month	20	11
at 3 months	90	50

When the distribution of titres is studied there is no significant difference between the groups. (For sera collected 1 month after inoculation $0.4 > P > 0.3$; for those taken at 3 months $0.3 > P > 0.2$)

This does not provide evidence that the formation of B/Taiwan antibody is suppressed by the presence of pre-existing B/England/59 antibody.

Neutralization Tests

Neutralization tests were carried out to determine the relation between the titres obtained by this method and by H.I. tests and, in particular, to discover whether the low titres to B/England/939/59 were caused by the low avidity of the test strain of virus rather than being a true reflection of the antibody present initially and after vaccination.

As a result the sera tested - all from Trial 1 - were not selected at random but mostly to test the validity of low titres.

One hundred and forty-seven sera from 37 individuals were tested with A/Singapore/1/57 and 419 sera from 105 people with B/England/939/59. In order to conserve tissue cultures an attempt was made to select likely dilutions by extrapolation from the H.I. titres. It was expected that the neutralization titres would be higher. This prediction was not always successful and thus some results were obtained only as "more than" or "less than" the nearest dilution tested.

The results are presented in the form of diagrams of points comparing the neutralization and H.I. titres of the same serum. Where a neutralization titre has to be expressed as "more than" or "less than" an arrow points in the appropriate direction. Where there are several sera in this situation the number involved is ringed.

The method of the test is shown in Appendix 4.

Figures 37 and 38 represent the results with A/Singapore/1/57 and B/England/939/59 respectively. In the former there is a fairly clear relation between the neutralizing and H.I. titres. A few sera that showed no antibody with H.I. had detectable neutralizing antibody. In general, the titres of neutralizing antibodies were found to be rather higher. However, titres of neutralizing antibody were read from the final serum dilution (i.e. in the virus/serum mixture). Titres of H.I. antibody were

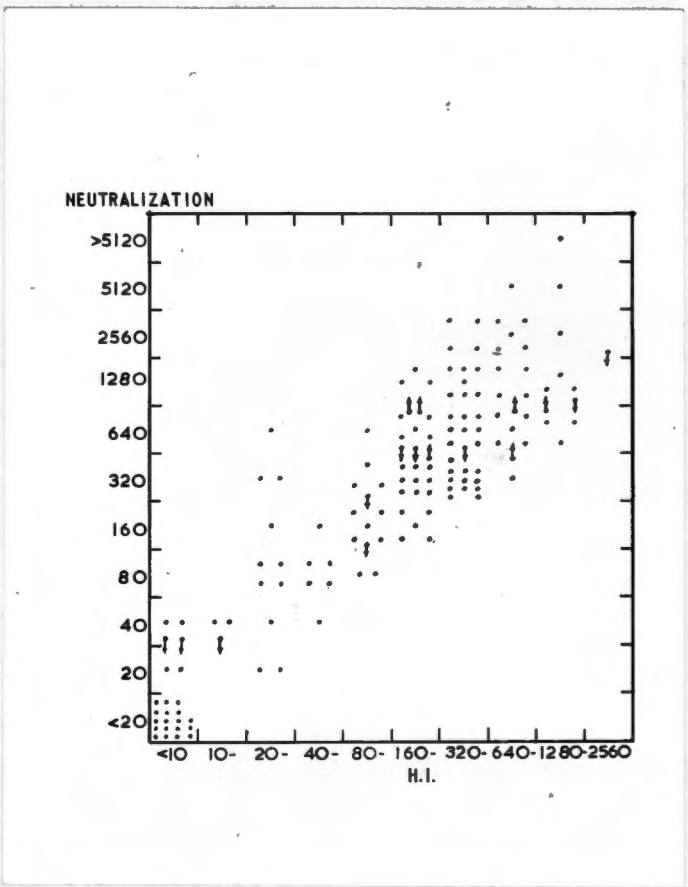


Figure 37. Diagram comparing titres of H.I. and neutralizing antibody to A/Singapore/1/57 found in the same sera.

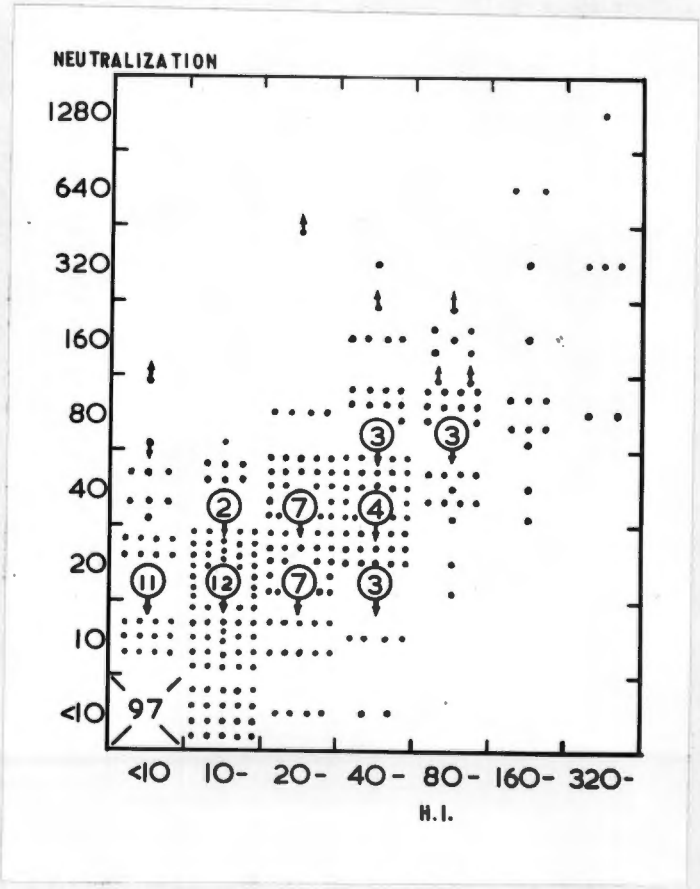


Figure 38. Diagram comparing titres of H.I. and neutralizing antibody to B/England/939/59 found in the same sera.

read at the level of the initial serum dilution.

In the case of the influenza B strain the relation is less clearly shown, partly because the readings are so much more concentrated at the lower end of the scale. However, there is a fair measure of agreement. If those sera with an uncertain titre are excluded, each test shows an approximately equal number of less than 10 readings where positive results occur with the other test. Certainly, the results seem to indicate that the H.I. titres generally represented true readings and that the results were not artificially depressed by the use of a non-avid strain in the H.I. tests.

SECTION D

RESPONSES OF ANTIBODY TO GROUP A SUBSTANCE

As the article by Springer and Tritel (1962), which stimulated this section of the study, only came to view after the start of the first trial any investigations to examine the effect of the vaccines on antibodies to Group A substances had to make use of stored sera. In view of this, and the fact that Springer and Tritel had investigated anti-A isoagglutinins, it was decided to examine the sera collected during the first trial to find the effect of the vaccines upon these antibodies. As a result of the findings further investigations were carried out on a limited number of the sera.

Because it was felt desirable to examine for the presence of antibodies that were associated with immunization with Group A substance it was decided to test sera obtained in the second trial for the presence of anti-A_1 haemolysins. Because of the high proportion of normal sera that contain anti-A_1 lysins (Lockyer and Tovey, 1960) and because of the apparent association with ABO haemolytic disease shown (Tovey, Lockyer, Blades and Flavell, 1962) with the presence of lysins to certain pig erythrocytes, tests for lysins active against A^p cells (Saison, Goodwin and Coombs, 1955) were also set up.

In addition, blood specimens were collected from

patients attending the Casualty Department of the Royal Victoria Hospital, before and 10-30 days after they had received prophylactic doses of anti-tetanus serum (A.T.S.). It has been shown that haemolysins to A_1 cells (Crawford, Cutbush, Falconer and Mollison, 1952) and A^p cells (Lockyer, 1963) are stimulated by certain batches of this substance and it was felt that the sera from these patients would act as a check upon the sensitivity of the test procedures used.

Programme

Trial 1

1. Blood was taken for grouping from all volunteers in the second part of the trial.
2. The sera of all Group O and B volunteers from whom four serum specimens (W, X, Y and Z) were available were examined for the titre of anti-A_1 saline isoagglutinins.
3. Of these volunteers, all that showed more than two fold rises of titre of iso-agglutinins, all that showed traces of haemolysis during iso-agglutinin titration and others that showed high iso-agglutinin titres, to a total of 50, had a pair of sera (W and Z) tested with added complement for haemolysins to A^p (porcine) cells.
4. Most of the volunteers who had haemolysins to A^p cells were given a further dose of influenza vaccine and serum samples taken before and after this were tested for

the presence of haemolysins.

Trial 2

1. After each bleeding session fresh serum specimens from all volunteers were tested for the presence of haemolysins to human A_1, A^p and O^p, erythrocytes at room temperature and at 37° C. The use of O^p cells (Appendix 6) was dictated by the need to differentiate lysis due to anti-species activity from lysis directed against that component of Group A cells which seems to be associated with ABO haemolytic disease.

Fresh sera were used so that there would be sufficient complement present for lysis to occur without the need of adding a further serum as source of complement and thereby introducing yet more variable factors.

2. All the stored serum specimens of Group O and Group B volunteers were tested for the titre of anti-A_1 iso-agglutinins.

3. During the period of the trial samples of serum from some patients attending the Casualty Department at the Royal Victoria Hospital were tested for the presence of haemolysins to human A_1, A^p and O^p cells before and at various periods after receiving anti-tetanic serum.

4. Over the winter, serum specimens from all Group O donors bled by the Northern Ireland Blood Transfusion Service were screened at 37° C. for the presence of anti-A_1

haemolysins.

The laboratory tests in this section of the work were carried out at the Northern Ireland Blood Transfusion Service Laboratory by Mr. T.E. Wilson, F.I.M.L.T. and Mr. F. Corr, A.I.M.L.T.

Materials and Methods

Sera

Blood specimens from the volunteers were taken as previously described. Where sera were to be tested for the presence of haemolysins precautions were taken to preserve complement. The majority were separated from the clot and tested in less than 8 hours after being drawn. A few were kept overnight at 4° C. Where a longer delay seemed inevitable the sera were separated and stored at -70° C.

Blood samples from the patients in the Casualty Department were taken by members of the staff there. Many of the patients did not return for a second specimen to be taken. Although disposable syringes were supplied several of the specimens were so severely haemolysed that tests for haemolysins were very difficult and only a proportion of the patients were Group O or B. These factors reduced the numbers of sera available.

Wherever it was possible, as with specimens from the vaccine trial, sera were tested for haemolysins in a

single batch in order to reduce variables. However, there were always some absentees who had to be bled, and their sera tested, at a later date.

Cells

The manner of collection and source of red cells used in various tests is described in Appendix 6.

Tests

The technique of tests performed in this section of the work is described in Appendix 6.

Results

Trial 1

(a) Of the volunteers from whom all four sera (W, X, Y, Z) were available, 207 were Group O or B. These were titrated for the presence of saline anti-A_1 iso-agglutinins. These results were examined to determine those volunteers in whom the titre -

i fell two-fold

ii rose two-fold

iii rose more than two-fold

iv did not alter as much as two-fold

Strict criteria for recognising an alteration in titre were adhered to and the two-fold change had to be clear. This was necessary owing to the drawn out end-points which occur when the agglutination end-point was determined microscopically.

Changes of titre associated with receiving vaccines W and R' could be compared between sera W and X (3 weeks), X and Y or W and Y (2 months).

Sera	W and X		X and Y		W and Y	
Vaccines	W	R'	W	R'	W	R'
Titres fell 2-fold	5	2	1	0	5	1
No clear change	75	88	87	93	79	75
Titres rose 2-fold	17	15	7	17	11	28
Titres rose 4-fold	0	3	1	0	1	4
Titres rose 8-fold or more	0	2	1	0	1	2

There was no significant difference in the changes between the vaccines at 3 weeks ($0.95 > P > 0.90$) or between 3 weeks and 2 months ($0.3 > P > 0.2$). However, there were significantly ($0.005 > P > 0.001$) more rises in titre with R' vaccine than placebo when the period November to January (sera W to Y) is considered. It will be appreciated that the changes of titre listed above cannot be simply added as minor variations in the titres not amounting to a clear two-fold change in themselves could still alter the number of valid changes recorded.

After serum Y (January, 1963) the administration of another vaccine complicated the situation and four different vaccine groups had to be considered between sera Y and Z.

Vaccine groups	WO	WR	R'O	R'R
Titres fell 2-fold	0	0	2	0
No change	40	43	48	52
Titres rose 2-fold	3	10	2	5
Titres rose 4-fold	0	1	0	1
Titres rose 8-fold or more	0	0	0	0

This distribution falls just outside the bounds of chance ($0.05 > P > 0.025$). Whereas there is no significant difference associated with the nature of the first inoculation received when comparing vaccine groups WO and R'O ($P = 0.64$) or WR and R'R ($0.2 > P > 0.1$), there are significantly more increases of titre associated with aqueous vaccine, R, than with adjuvant. This is shown when the combined results of WO and R'O are compared with WR and R'R combined ($0.025 > P > 0.01$).

(b) When the 50 pairs of sera (W and Z) which had shown lysins or suspicious iso-agglutinin titres were examined for haemolysins to A^p red cells only 4 sera, all Z, produced even a trace of haemolysis. In this test, unlike others described later, papainized A^p cells were used and the test run for 1 hour at room temperature.

Of these 4 students 3 were males and a further dose of 1.0 ml. of aqueous (R) influenza vaccine was administered and serum taken before the inoculation and stored at -70° C. was compared with serum taken 11 days

after inoculation. Of the second specimen part was snap-frozen to -70° C. and thawed to make the comparison of the two samples more exact. This extra inoculation was given 9 months after the previous dose of vaccine which they had received.

All 3 volunteers had haemolysins to A^p cells in both serum specimens. Two also had lysins to O^p cells which suggested that the reaction could be merely anti-species activity. However, two of the three possessed anti-A_1 haemolysins. The Antibody-Absorption test showed all three to have weak or moderate positive results. However, none of these results were affected by the inoculation. The lysins found in the sera taken before their booster inoculation were fractionally more pronounced than those found in the sera taken after this injection.

Trial 2

(a) Of 97 volunteers in the second trial 52 were Group O or B. Only 51 fresh sera were available at 3 months as too little blood was obtained from one volunteer.

i Haemolysins

As it was necessary to use fresh sera the tests were carried out soon after the sera were taken. Because of this the 3 sera of each person were not tested together and thus results are more strictly comparable in the mass, than when comparing individuals. However, results were

generally consistent.

Although tests for haemolysins were carried out both at 37° C. and room temperature, so few lysins were shown at room temperature that only the results at 37° C. will be considered.

The results of the findings on fresh sera are shown below.

Cells	Human A_1			A^p			O^p		
	C	P	T	C	P	T	C	P	T
Sera									
Before (F)	0	2	0	3	15	1	2	12	0
1 month (G)	0	1	0	1	22	0	0	18	0
3 months (H)	0	1	4	1	23	11	1	22	17

C - Complete Haemolysis

P - Partial Haemolysis

T - Trace of haemolysis

At the kind invitation of Dr. G.H. Tovey, sera were sent to the South-West Regional Blood Transfusion Centre, Southmead, Bristol, to check these results. Here the sera were absorbed with O^p cells to remove anti-species activity before being tested against A_1 and A^p cells. In addition, the sera were tested for the presence of complement with sensitized sheep cells. Some discrepancies with the results obtained in Belfast were noted. The results from Bristol have added the following

records of lysins to the table:-

Cells	A_1			A^p		
	C	P	T	C	P	T
Sera						
Before (F)						1
1 month (G)		1	2		4	
3 months (H)	3		1	2		

Repeated efforts were made in Belfast to confirm these results. As the sera had, by now, been stored at -20° C. for months all sera were tested for complement with sensitized sheep cells. In parallel tests fresh, lysin-free, human Group O serum was added as a source of extra complement. It was not possible to confirm the existence of the complete lysins found in Bristol in any of the sera. In the post-inoculation sera of one volunteer the presence of trace levels of haemolysin to A_1 cells was confirmed, having not been detected during the examination of the fresh sera.

ii Iso-agglutinin titres

In the absence of a control series the findings of iso-agglutinins were of less value. Here the titres were recorded and compared.

Changes in titrè are recorded in the table below.

Sera compared	F - G	G - H	F - H
Fell 4-fold	1	0	1
Fell 2-fold	3	1	3
No change	40	39	30
Rise 2-fold	7	9	14
Rise 4-fold	1	3	3
Rise 8-fold or more	0	0	1

(As mentioned above, there was too little serum from one volunteer to perform tests for lysins. By diluting the amount available an approximate iso-agglutinin titre was measured and this is included in the results. This showed a two-fold increase over the previous sample.)

(b) Casualty patients

Pairs of sera were available from 16 people who were Group O or B. Seven of these had been bled 9-15 days after receiving 1,500 units of A.T.S. and 9 a month after their inoculation. The initial serum of one of the first group and two of the second were so haemolysed as to make it impossible to test for lysins. Lysins were detected so much more frequently in this group that a comparison between the results obtained from tests at room temperature are more revealing than those at 37° C.

	Room Temperature						37° C.					
	Before A.T.S.			After			Before A.T.S.			After		
	A^p	O^p	A_1	A^p	O^p	A_1	A^p	O^p	A_1	A^p	O^p	A_1
1/ 10 day interval												
Trace	0	1	0	0	0	0	0	0	0	0	0	0
Partial	0	0	0	0	2	3	2	2	0	0	1	4
Complete	2	0	1	4	1	1	2	1	2	6	4	2
2/ 1 month interval												
Trace	0	0	0	0	0	0	0	0	0	0	0	0
Partial	1	0	0	4	0	1	4	0	1	1	4	5
Complete	0	0	0	2	0	1	2	0	1	6	0	1

On the serum specimens of two of these patients, one from each group, an Antibody-Absorption test was carried out. Both had shown acquired haemolysins at A_1 cells at 37° C. following A.T.S.

		Pre-absorption Titre	Post-absorption Titre
1/ 11 day interval	Pre-A.T.S.	32	8
	Post-A.T.S.	512	8
2/ 30 day interval	Pre-A.T.S.	32	8
	Post-A.T.S.	128	8

This seems to indicate that the increase in titre has been primarily due to the increase of the anti-A^p component.

The volunteers for the influenza vaccine trial had been questioned about past injections of anti-tetanic serum. Of those who were Group O or B, 4 had received A.T.S. within two years and 2, 4 and 5 years previously, respectively.

These numbers are small but there appears to be no tendency for the potency of lysins present in these people to be increased following influenza vaccine.

		Pre-inoculation			1 month			3 months		
	Temp.	A^p	O^p	A_1	A^p	O^p	A_1	A^p	O^p	A_1
A.T.S. within 2 years										
505	R.T.	-	-	-	-	-	-	-	-	-
	37°	P	-	-	P	P	-	T	T	-
562	R.T.	P	P	-	P	P	-	P	P	-
	37°	C	C	-	P	P	-	P	P	-
563	R.T.	P	-	T	P	-	-	T	-	T
	37°	P	-	P	P	-	-	P	-	T
608	R.T.	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	P	-	-
A.T.S. 4-5 yrs. ago										
543	R.T.	-	-	-	-	-	-	-	-	-
	37°	P	T	-	-	-	-	P	-	-
601	R.T.	-	-	-	-	-	-	-	-	-
	37°	-	-	-	-	-	-	P	-	-

C - Complete, P - Partial, T - Trace Haemolysis.

(c) Haemolysins in Donor Sera

In order to provide an index of the normal incidence of anti-A_1 lysins in the population of Northern Ireland the routine test used for screening Group O donor blood was brought to the same standards as that used in the trial and results were recorded for each donor bleeding session. Pig cells were not used.

The relevant results from the vaccine trial volunteers are included for comparison. Whereas the donors are only Group O the volunteers have Group B people included.

Month		Haemolysins			No Lysin	Totals
		Complete	Partial	Trace		
1963 Nov. (26th-29th for donors)	Donors	0	16	38	288	342
	%		(4.7)	(11.1)	(84.2)	
	Volunteers	0	2	0	50	52
	%		(3.8)		(96.2)	
Dec.	Donors	1	52	166	1276	1495
	%	(0.1)	(3.4)	(11.1)	(85.4)	
	Volunteers	0	1	0	51	52
	%		(1.9)		(98.1)	
1964 Jan.	Donors	0	37	146	1704	1887
	%		(2.0)	(7.7)	(90.3)	
Feb.	Donors	0	43	112	1351	1506
	%		(2.9)	(7.4)	(89.7)	
	Volunteers	0	4	1	46	51
	%		(7.8)	(2.0)	(90.2)	
Totals	Donors	1	148	462	4619	5230

CHAPTER IV

DISCUSSION

REACTIONS TO INFLUENZA VACCINES

In the trials constituting the present study local reactions were much more prominent than systemic ones. From the first part of the first trial the association of symptoms with even the small dose of vaccine was established. It also appeared that the effect of vaccine, as distinct from that of the injection, took some hours to develop.

It is difficult to understand why erythema was less frequent with 1.0 ml. of aqueous vaccine than with 0.5 ml. of the same material 2 months previously. It is possible that the batch of vaccine, prepared for the trial, had had the extra period to age and lost some of its toxic properties. This is commonly thought to happen with some bacterial vaccines although no proof exists. If this is so the erythrogenic property seems to be somewhat more labile and, at least, partly separate from the other toxic properties because pain and tenderness were not noticeably affected by the period of storage. This, in turn, would afford an explanation why the presence of erythema, following sub-cutaneous inoculation, was not necessarily correlated with other symptoms. When volunteers receiving

saline vaccine were considered the incidence of "Pain" graded at more than "½" was similar in those with and those without erythema (16.6% and 15%). However, the incidence of "Tenderness" graded more than "½" was twice as high in those who had erythema (25%, as against 12.5%).

It is also likely that the relative absence of erythema following adjuvant vaccine is related to the deep site of inoculation as much as to the smaller amount of antigen contained in a dose.

The site of inoculation would also affect swelling and induration. The incidence of these was small and as, like erythema, these symptoms are not disabling in themselves, it would be misleading to employ these as main criteria of local reactions (M.R.C., 1955) although they have the advantage of being objective findings and all three were associated only with vaccine and not with the placebo.

The degree of pain recorded would clearly be related partly to the temperament of the recipient of the vaccine. However, it is also clearly related to the mass of influenza antigen, as the incidence was much higher with the 1.0 ml. dose of aqueous vaccine than with 0.5 ml., and to the site of inoculation. Although adjuvant vaccine contained so much less antigen than the aqueous dose the incidence of pain was only slightly less and the duration of symptoms was longer. Probably this is partly related

to the fact that muscular movement would irritate the site of intramuscular inoculation while the sub-cutaneous tissue is unaffected. That the nurses, more frequently engaged in manual work, were more affected in Trial 2 than the students would tend to support this contention.

Tenderness lasted equally long with both aqueous and adjuvant vaccines. Certainly the amount of tenderness with adjuvant vaccine is more than would be expected from the dose of antigen it contains. The ethical position of inoculating volunteers with an oily adjuvant containing only placebo would be dubious but without such a control group it was difficult to gauge how much tenderness was due to the reactive process about the mineral oil droplets and how much due to the influenza antigen.

The greater incidence of bruising among those receiving adjuvant vaccine was probably due to the use of the heavy cartridge syringe and the deeper inoculation. In the second trial the large needles used for the majority of the nursing staff might well explain the number and persistence of bruises in this group. These bruises were not a cause of complaint.

After the satisfactory absence of any definite residual lesions following adjuvant vaccine in the first trial the occurrence of nodules in 4 volunteers in the second trial was surprising and disappointing. In all

cases the lesions were clearly in the deep sub-cutaneous tissue so either the inoculum had never been placed in the belly of the muscle or it had leaked back along the needle tract. As the long head of the triceps is fairly deeply situated the former is not unlikely. It would seem that this site was not a satisfactory one for the inoculation and while the deltoid is a much poorer area for assessing reactions in a trial of this nature, it is undoubtedly a better site for routine use.

The histology of the reaction about the oily adjuvant in the monkey experiments was that of an oil granuloma and was similar to the description of Humphrey and White (1963). It is interesting that in the sub-cutaneous inoculations both in the 4 volunteers and in the animal experiments the large thin-walled cysts described by Salk and Laurent (1952) did not develop. This is possibly related to use of less toxic and purified oils and emulsifying agents.

Systemic reactions were not prominent. The cases of allergic types of reaction are not convincingly linked with the vaccine. The one florid attack of urticaria occurred in a student given placebo. Examining the cases individually possibly one student who had urticaria in the first part of the trial might have been affected by the vaccine but a later dose of adjuvant vaccine, as mentioned

above, did not cause any allergic manifestations. It would seem that the precautions of excluding sufferers from egg hypersensitivity and active asthma were sufficient.

No definite pattern of other systemic symptoms developed in conjunction with the vaccines. In the first part of the trial as many, or more, reactions of all kinds occurred with placebo as with the vaccine. In the second part symptoms occurred more frequently in those given aqueous vaccine with 15,000 H.A. units of virus than with 3,000 H.A. units of virus in the adjuvant vaccine which might be significant but even here the numbers are so small that this is within the bounds of chance. The incidence of cases with definite malaise or shivers in the group receiving aqueous vaccine was just over 1%.

Coryza in the first 36 hours after inoculation occurred more frequently in those receiving aqueous vaccine than those getting adjuvant in the second part but more frequently after placebo than vaccine in the first part.

In the second trial the position is more difficult to assess in view of the smaller numbers, the lack of a control group and fewer examinations but here again no definite pattern appeared although the feeling of faintness in one student and the cold complained of by a

nurse might have been associated with the vaccine.

Generally speaking, the lack of systemic reactions following the vaccines used in these trials was in sharp contrast to an incidence of 15% of symptoms of malaise and shivers in a class of students who were given 0.2 ml. of Typhoid-Paratyphoid A and B-Tetanus Vaccine intradermally.

In agreement with the report of Sadusk et al., 1949, females were affected more than males throughout. This occurred with the objective signs, like erythema, as well.

It is interesting that the subjective questioning did not reveal any preference among the inocula. From the first part of the first trial it seemed that the assessment by the volunteers was a less sensitive gauge of reaction than the examinations. This is, perhaps, not surprising because the system employed must have discovered nearly every reaction while complaints would only have been elicited by reactions above a certain threshold. However, both sets of questions, on the acceptability of the vaccines or comparing the reactions, showed that the full doses of vaccine had felt worse and a significant number felt that they would not readily accept the latter vaccines. It was a pity that the reasons were not collected.

Pain and tenderness were the most prominent symptoms recorded. If it is arbitrarily presumed that a record of these graded as more than "½" would merit a complaint the

the incidence of local reactions were not strikingly different from those reported by Clarke (1962).

	Tenderness	Pain
Placebo	3.8%	10.7%
R'	20;8%	13.1%
R	34.2%	29.3%
O	24.0%	20.5%

Clarke (1962) Local Reactions 32%

However, the incidence of systemic reactions and of local reactions which interfered with activities during all phases of the present studies was in striking contrast to many of those previously reported and, in particular, to those reports of Clarke (1962) and Meichen et al. (1962) which concerned British types of vaccine.

None of our volunteers felt that they had missed work, play or social activities through the vaccine. In the series investigated by Meichen et al. (1962) 10-15% were unable to do a normal day's work on the day following vaccination. While systemic effects were rare in the present series, Clarke (1962) reported that 30% of the recipients suffered from general malaise and, again a particular difference, that 6% suffered from allergic complaints. Obviously, the order of reactions experienced by these workers was quite different from that observed in our trials which featured multiple examinations but also

different vaccines and controls unknown to the volunteers. It is difficult to reconcile these differences adequately.

However, while those responsible for the development of vaccines should never allow themselves to be entirely satisfied with the current product - a self-satisfaction that has delayed much-needed improvements in several vaccines - the acceptability of a vaccine by the general public is geared principally to the fear engendered by the severity and imminence of the disease in question and partly to the reaction caused by it. Leipoldt (1937) has commented on the ambivalent attitude of the public towards diseases - dreading the dramatic and scorning the familiar, regardless of which actually causes more harm. Influenza is both familiar and intermittent and has a low case-fatality rate. It is little feared. Rosenstock (1961) described the results of a survey made among 1,600 families in the summer of 1957 on the eve of the Asian influenza epidemic. Despite the massive publicity that influenza was receiving at the time only 25% of the families interviewed accepted the possibility that someone in the family would catch influenza. Less than 3% believed that an attack of influenza would cause them to alter their daily activities.

Modern medicines are not always bitter. The inoculation of Salk-type poliomyelitis vaccine was

particularly devoid of reactions. It could well be reasoned that a prophylactic against influenza should cause no worse reaction than that!

Although the vaccines used caused little interference with activities the incidence of pain and tenderness was not negligible. In children the degree of reaction would, undoubtedly, have been worse. While the present vaccine would be quite acceptable for groups of individuals with chronic pulmonary or cardiac disease who would particularly fear an attack of influenza the numbers of volunteers who felt that the vaccines would not be worth while might indicate that a more bland preparation is desirable.

Hoyle (1952) showed that the influenza virus particle could be fragmented by ether and that the nucleoprotein moiety could be precipitated by lanthanum acetate. Experiments of Davenport, Rott and Schäfer (1960) showed that the haemagglutinin fraction was sufficiently antigenic and Mizutani, Beals, Hennessy and Davenport (1962) indicated how a satisfactory amount of a haemagglutinin fraction could be prepared using lanthanum acetate. This purified haemagglutinin fraction extracted from formalinized virus was proved (Davenport, Hennessy, Brandon, Webster, Barret and Lease, 1964) to be effective in eliciting antibody and very considerably less prone to

cause reactions than intact vaccine, even in small children. It is possible that a vaccine of this nature could effectively reduce the incidence of reactions to a still smaller degree.

ANTIBODIES TO GROUP A SUBSTANCE

Haemolytic disease of the newborn due to anti-A is felt to be a common syndrome (Halbrecht, 1944, Hsia and Gellin, 1954), occurring at least 1:150 births, but it appears to be a mild condition in the vast majority of cases and rarely requires treatment (Mollison, 1956). Even the criteria for treatment are different from those with haemolytic disease due to the Rh factor (Tovey, Gillespie, Guy, Valaes, Oppé and Lewis, 1959). It has been shown that where a pregnant woman is Group O and her infant is Group A the mother's anti-A iso-agglutinin titre frequently rises to considerable heights in the post-partum period (Boorman, Dodd and Mollison, 1945) and the thermal amplitude of the anti-A agglutinins also changes (Hubinont, 1949).

It has been clear for a number of years that the inoculation of various therapeutic substances including bacterial vaccines (e.g., Typhoid-Paratyphoid A and B vaccine) and anti-tetanic serum frequently induce anti-A haemolysins in Group O recipients (Crawford et al., 1952).

The parenteral administration of Rh substance, whether by injection, transfusion or previous pregnancy is known to sensitise susceptible women to this substance and to increase the chances of haemolytic disease of the newborn in a subsequent pregnancy.

The results obtained in the present study following the administration of influenza vaccine must be discussed in the light of these findings.

Following the initial inoculation of the first trial there was a slight and delayed tendency for titres of anti-A_1 iso-agglutinin to rise. Whereas a 2-fold change of titre is not significant when comparing two individual specimens, due to the degree of variation inherent in the test, in this case changes of titre were not randomly distributed between rises and falls and the tendency to rise, judged by these slight changes, was valid. The fact that there was a rise, although smaller, in the titres of the group given placebo indicates that another factor besides the vaccine was operative. In the second part of the trial the upward tendency of the iso-agglutinin titres was greater in those getting aqueous vaccine, with the larger antigenic mass and a rise was also evident in the second trial.

While this increase was in agreement with the general statement of Springer and Tritel (1962) the degree and

uniformity of the change was quite different.

The significance of this increase of iso-agglutinins in relation to disease is also open to question. The use of different tests discloses a wide range of different antibodies to A-substance. However, whereas saline iso-agglutinins with optimum activity at 4° C. occur naturally, antibodies with properties of haemolysis (Crawford et al., 1952), optimal activity at 37° C. (Hubinont, 1949) or of activity after partial neutralization (Witebsky, 1948) are formed in response to immune stimuli.

Tisdall, Garland, Szanto, Hand and Bennett (1946) showed that the titre of haemolysins present in a serum is usually proportional to the titre of agglutinins but numerous exceptions to this have been recorded (Ervin, Christian and Young, 1950, Grove-Rasmussen, Shaw and Marceau, 1953, Stevens and Finch, 1954) and the instances of transfusion reaction that are described by these authors confirm that the dangerous qualities of sera from Group O donors to Group A or B recipients do not reside in the natural anti-A or anti-B iso-agglutinins but in antibodies showing "immune" characteristics. Regarding haemolytic disease of the newborn the importance of natural saline iso-agglutinins must be small as the placenta is relatively impermeable to the large molecular

globulins which comprise these antibodies (Tovey, 1945).

Not all the tests for "immune" type anti-A antibodies are equally applicable to large numbers of sera and tests for haemolysis are the easiest to carry out (Crawford et al., 1952) as well as being a good index of these antibodies (Davidsohn, Goodman and Stern, 1956). L.A.D. Tovey (1957) felt that whereas a serum containing an incomplete antibody will almost invariably contain a haemolysin the reverse does not hold so that tests for haemolysins will show a maximal incidence of relevant, immune-type, antibodies. The work of Gardner and Tovey (1954) supports this contention. The incidence of anti-A_1 haemolysins in serum from Group O donors has been found to be so high by several workers (Chaplin, Wallace and Chang, 1956, McDermott and Muschel, 1956, Lockyer and Tovey, 1960) that if the presence of these lysins was used as an index an excessive amount of Group O blood would be discarded as being dangerous for emergency use in Group A or AB recipients. As a result Lockyer and Tovey (1960) developed the test for lysins to A^p cells (Winstanley, Konugres and Coombs, 1957) to provide a screening test of more reasonable sensitivity.

In the present study the technique adopted provided degrees of sensitivity ranging from the detection of anti-A_1 haemolysins at 37° C. to the detection of anti-A^p

lysin active at room temperature. This would have provided for different levels of incidence of haemolysin in the pre-inoculation sera. In the event, the most sensitive index proved to be the most useful although it was complicated by non-specific lysis of pig cells at 37° C. (Tovey, Lockyer, Blades and Flavell, 1962).

The incidence and potency of anti-A_1 haemolysins occurring following the inoculation of influenza vaccine in Trial 2 is exceedingly low and well within the bounds of natural variation found (Tovey, L.A.D., 1957). It is difficult to explain the discrepancies between the results obtained in Bristol and Belfast. The sensitivity of tests for lysins is governed both by the concentration of complement present and the concentration of the cells used (Chaplin et al., 1956). However, the concentration of cells used in Bristol (4%) was only slightly less than that used in Belfast and the test in Belfast employed either fresh sera or added complement. It is of possible value that those cases about which there is lack of agreement did not show significant increases in saline agglutinin titres. However, while these discrepant results are a source of doubt they do not alter the situation that the influenza vaccine used did not show a definite tendency to stimulate immune type antibodies to Group A_1 cells. The results are markedly different from

those found by Crawford et al. (1952) with bacterial vaccines, where 10 of 14 recipients acquired haemolysins after Typhoid, Paratyphoid A and B vaccine.

It is also of interest that, in agreement with Springer and Tritel (1962), there was no evidence that the titres of anti-A_1 saline agglutinins were enhanced by a repeated inoculation. This was shown both by the lack of difference, in the first trial, between the responses to the second injection of those given placebo or vaccine initially and in the lack of response of the three volunteers given a further dose of vaccine. In addition, in the second trial, influenza vaccine did not appear to stimulate any special response in those volunteers who had previously received anti-tetanic serum. Therefore, it is less likely that repeated doses of influenza vaccine will elicit antibodies not detected in the present study.

Because of the low overall incidence of anti-A_1 lysins in the serum of Group O donors in Northern Ireland it has been suggested (Tovey, 1963) that the population is inherently resistant to antigenic stimuli of this nature. If that were so the results of the present work might not be universally applicable. This suggestion is difficult to disprove without further work but the higher incidence of lysins in the rather special group comprising the casualty patients and their response to anti-tetanic serum would

tend to indicate both that the tests employed were sufficiently sensitive and that an inherent resistance to stimuli of Group A substances is not present.

It is beyond the competence of this study to provide evidence as to whether ABO haemolytic disease of the newborn is in fact stimulated by previous experience of Group A substance by the Group O mother of susceptible children. However, the high proportion of cases occurring in first-born children, estimated as 40-50% (Levine, Vogel and Rosenfield, 1953, Mollison, 1956) and the absence of the disease in a susceptible infant following severe disease in a previous pregnancy reported by Crawford, Cutbush and Mollison (1953) do not seem to support this. In his survey, Tovey (1957) also found no consistent factor, such as previous inoculations, that could be incriminated as a cause of this condition. It is clear, however, that only large-scale prospective trials could settle this point satisfactorily.

INFLUENZA ANTIBODY RESPONSES TO VACCINE

The results obtained from a trial of a vaccine need to be considered relative to certain requirements.

Was the antigenic stimulus sufficient to elicit an adequate antibody response?

The median titres appeared to indicate that 0.5 ml. of aqueous vaccine produced as high a titre of antibody to A/Singapore/1/57 as 1.0 ml. of vaccine and, also, that the titres reached after the small dose were not augmented by a further, larger dose. However, a study of the response of antibody to B/England/939/59 shows a rather different pattern.

If the volunteers in Vaccine group R'R are divided into two groups, one of those whose initial serum had no detectable H.I. antibody to B/England/939/59 and the other of those with an initial antibody titre of 10 or more the evolution of antibody titres after the doses of Vaccine was as follows:-

Initial levels		10	10 or more
		Median titres	
Serum	W	10	20
	X	15	60
	Y	15	60
	Z	27.5	60

Several points are illustrated by this.

1) Where an individual has had previous experience with an influenza antigen a small dose of vaccine is sufficient to cause a rise of antibody titre to a "ceiling" at which a further dose of vaccine does not cause any further rise. This was the case with A/Singapore as nearly all the volunteers had antibody to this strain in their pre-inoculation serum.

This tendency for vaccination to bring antibody levels up to a "ceiling" has been frequently noticed in the past and has been shown to be responsible for the difficulty in making a serological diagnosis of influenza in people who had been vaccinated recently (Horsfall et al., 1941b, McDonald and Andrewes, 1955). Beveridge (Beveridge, 1944b, Beveridge, Stone and Lind, 1944) attributed this to the effect of existing antibody reducing the antigenicity of subsequent doses of virus.

2) That where individuals had not had previous experience with an antigen the small dose did cause a rise in antibody but only to a low level and a further rise was stimulated by a second inoculation. While the timing of the doses was probably not optimal, the median titre produced by even two doses was not high.

The relative inadequacy of the half dose of vaccine was further indicated by comparing the ability of the different doses to induce antibody in those who had none initially. No detectable antibody was found in 25% of those

receiving 0.5 ml. of vaccine and in only 7% of those getting a dose of 1.0 ml. of the same vaccine.

Thus, there is evidence that while 0.5 ml. of vaccine was sufficient in those who already had antibody to the antigens it was inadequate for people without resting antibody.

The graphs showing median titres at 9-10 months show that two doses of vaccine did not appear to cause better persistence of antibody levels than only one inoculation.

When incorporated with mineral oil in a water-in-oil emulsion it appeared that $^{1}/_{5}$ of the amount of antigen gave rather similar antibody titres to the full dose of antigen in an aqueous medium.

Some differences in the antibody response between the aqueous and adjuvant vaccines appeared. With influenza B it appeared that the effect of the adjuvant vaccine was delayed more than the aqueous vaccine. At 4 weeks the median titre of this group was lower than that of the group receiving aqueous vaccine. However, at 9-10 months the position was reversed and the median titre of the group receiving adjuvant vaccine was higher at this time than at 4 weeks. In the second trial the distribution of titres at 1 and 3 months shows that antibody formation was delayed in those with no previous experience of the antigen but that this difference had partly disappeared at 3 months. However, unlike the aqueous vaccine, the adjuvant vaccine "O" seemed to be able to boost the titre of antibodies to A/Singapore

above the "ceiling" reached after 0.5 ml. of acueous vaccine.

While the second trial confirmed that the effect of adjuvant vaccine continued for more than a month, with antibody titres rising to considerably higher levels at 3 months, the first trial showed the superiority of the adjuvant vaccine over the aqueous vaccine at maintaining titres.

A very prominent feature was the marked difference between the antibody titres to A/Singapore/1/57 and B/England/939/59. This was also shown with all the strains of influenza B virus tested. While a much higher proportion of the volunteers had antibody to influenza A than to influenza B the table above shows that even in those who had antibody in their first serum doses of vaccine did not produce generally high levels of antibody to influenza B. It is difficult to propose a reason for this. If this low level of antibody was due to insufficient antigen in the vaccine why did half a dose bring the levels of those with antibody initially to their maximum? If it was caused by poor avidity of the virus strain used in the test (Isaacs and Andrewes, 1951) why was no great discrepancy shown by the neutralization test? If this was an inherent property of the influenza B virus why were much higher titres found in previous trials with 1940 and 1954 strains of influenza virus. (Francis et al., 1946, Davenport et al., 1962)?

Probably a combination of these factors was operating in this case. Likewise, a change of avidity of the test strain of B/England/939/59 during the course of passage in the laboratory was unlikely to have been the cause of the unduly low titres recorded in the early group of tests done as no evidence for this was found on examination. However, although it was impossible to test this retrospectively, the problem could have been related to the varied sensitivity of chicken red cells. Stuart-Harris (1943) and Hirst (1943) found that individual variations in the sensitivity of chicken red cells affected influenza B virus estimations particularly.

During the period covered by these two trials the only evidence of influenza came from an old people's home where there was a sharp outbreak of influenza A in March, 1962. In these circumstances it was not possible to judge whether the high titres of antibody to A/Singapore/1/57 or the low titres to B/England/939/59 were associated with protection.

Thus, a further requirement of these vaccines is contained in the problem of whether the titre of H.I. antibody elicited constitutes a reliable index of the efficacy of the vaccines in giving protection against the disease. From the time when the difference between the Swine and Human Type A viruses was recognized it has been felt that protection was associated with the strain specific antigen

that was measured by the neutralization test rather than the type specific soluble antigen which dominated the ordinary complement-fixation test (Fairbrother and Hoyle, 1937). While Hirst (1942a) showed a fairly close correlation between the H.I. test and a neutralization test in mice Walker and Horsfall (1949) demonstrated, by differential absorption, a lack of identity of neutralizing and H.I. antibody. Using randomized fragments of surviving chorio-allantoric membrane for a refined neutralization test Fazekas de St. Groth, Withell and Lafferty (1958) concluded that anti-haemagglutinin titres were not related to the neutralizing power of sera in any simple manner. Keeble (1963), working with inactivated Newcastle Disease vaccine, showed that the curves depicting the development and decline of neutralizing and H.I. antibody diverged considerably.

In the present study, while the neutralization test fulfilled its purpose of providing an estimate of the avidity of the viruses used in the haemagglutination test, it was not sufficiently presise for further conclusions to be drawn. Despite this the technique was laborious and not well suited for the examination of large numbers of sera.

The difficulties concerning the relationship between titres of circulating antibody and immunity to influenza have been mentioned earlier already. It has been shown

many times that clinical influenza tends to occur in those people with low serum antibody titres to the epidemic virus, including by Burnet (Burnet and Foley, 1940) and Meiklejohn (Meiklejohn et al., 1952a), and Salk, Menke and Francis (1945), comparing vaccinated and unvaccinated populations, showed that attack rates of influenza were equal in those of both groups with the same acute phase titres. However, Hare (1940), using a mouse neutralization test, felt that the serum antibody titre in humans gave no indication of susceptibility or immunity to infection. The studies of Horsfall (Horsfall et al., 1941b) and Hirst (Hirst et al., 1945) showing that high serum titres of antibody did not always confer protection, have already been mentioned. Trials reported by the Medical Research Council (1957) showed a lack of protection despite raised antibody titres but, in other circumstances, protection was shown despite a poor H.I. antibody response (M.R.C., 1958, Rose, 1961) !

Experiments on conferring passive immunity to mice showed the superiority of an intra-nasal route over an intraperitoneal route for the administration of immune serum (Stokes and Shaw, 1939, Taylor, 1941). However, influenza in the ferret is a much more comparable disease to that in man and Zellat and Henle (1941) felt that intra-nasal immune serum did not protect ferrets against normal influenza, only against pulmonary involvement. Burnet (Burnet, Lush and Jackson, 1939) and Francis (Francis, 1940a, 1941,

Francis and Brightman, 1941, Francis, Pearson, Sullivan and Brown, 1943) studied the presence of virus neutralizing substance in nasal secretions and the latter felt that this was antibody. However, as both workers inserted cotton wool plugs into the nostrils to stimulate and collect secretion it is possible that the resulting irritation may have produced a secretion not comparable to that found in the normal naso-phaxynx.

Fazekas de St. Groth and Donnelley (1950a and b) showed in experiments with mice that while no mice without circulating antibody showed resistance to influenza, serum antibody levels gave very little information about the specific protection afforded and that there was a strict correlation of immunity with the presence of haemagglutinin-inhibitor in the bronchial tract.

These experiments seem to indicate that resistance to influenza might be a function both of the titre of circulating antibody and of the permeability of the naso-pharynx whereby antibody might be present in the surface secretions. However, influenza in mice is a primarily pulmonary disease and, as has been mentioned above, ferrets, which, like man, contract a more superficial form of influenza were not protected from this antibody but only by an alteration of the nasal epilhelium (Francis and Stuart-Harris, 1938).

Thus while the estimation of H.I. titres of antibody

are convenient and afford a measure of the efficacy of a vaccine the results gained in this manner must be interpreted with some reservations in view of the imperfect correlations of H.I. and neutralizing antibody and of circulating antibody and protection.

What should the composition of influenza vaccines be? As the controversy over the fundamental concepts governing these vaccines is still unresolved it is clear that much difference of opinion exists. While Davidson (1961) suggested that a polyvalent vaccine, containing several strains of influenza A virus, might stimulate antibody to antigens of the past rather than to the new strain which was current, Jensen, Woodhour and Bailey (1960) claimed that the vaccine used in the U.S. Armed Forces containing 6 strains of virus was better than a 4 strain vaccine even when an equal "mass" (by C.C.A. units) of each was administered. The results shown above indicate that pre-existing B/England/939/59 antibody did not seem to interfere with the response to B/Taiwan/4/62 antigen. However, the significance of this is open to arguement as the antigenic relations between strains of influenza B virus are closer than between the sets of influenza A.

It is of interest that the addition of B/Taiwan/4/62 antigen to vaccine should also elicit antibodies to B/Lee and this unexpected observation made it necessary to test

sera from the previous trial to show that no such antibodies were produced by the vaccine containing only B/England/939/59. This result might either indicate that the increased amount of type B antigen resulted in a general rise of minor antigenic components or that there is some subtle relation between B/Taiwan/4/62 and B/Lee. Experiments with the convalescent ferret sera by Green, Hung, Yu, Lee and Pereira (1964) and with specific chicken sera in the present study show a progressive antigenic drift with the strains of influenza B so that B/Lee showed no crossing with B/Taiwan/62 strains although hyperimmune rooster sera (Green et al., 1964) did show some relation. However, there is no clear evidence of the "closing" of any putative "ring".

While there is controversy (Davenport, 1963) over the interpretation of the results, there are indications (Influenza Surveillance, 1963) from several studies that the polyvalent American vaccine failed to give adequate protection against the strains of Asian influenza that caused an epidemic in the United States during the winter of 1962-1963. If these studies are valid they might indicate that the amount of Asian antigen that could be incorporated in the polyvalent vaccine was inadequate, that the presence of type A antigens from other sets of virus (A and A_1) were of no assistance in making the

vaccine effective or that the degree of variation shown by the prevalent A_2 strain was too great for the earlier A_2 strain in the vaccine to extend protection - or any combination of these possibilities.

If any of the circumstances above are correct, and particularly the first two, doubt is case on the validity of the concept underlying the polyvalent, American-type vaccine. In the case of the third an interesting relation between antibody and protection may be present. The strains isolated from the American epidemic were similar to A/Netherlands/65/63. It has been shown above that certainly in a population with previous experience of A_2 strains of influenza - almost a universal state - a vaccine containing A/Singapore/1/57 and a variant of a different type, A/England/1/61, stimulated a considerable rise of antibody to A/Netherlands/65/63. Does that indicate that the strain specificity of protection can be, at times, more precise than that indicated by H.I. antibody? In fact, from the reports already quoted, this appears to vary and there seems to be no certain way of predicting whether a new, but related, strain needs to be incorporated into vaccines or not.

While Langmuir, Henderson and Serfling (1964) have called for a re-appraisal of American influenza vaccines there do not seem to have been adequate trials to compare

the efficacy of vaccines compounded on the British and American systems with regard to either the production of antibody or the prevention of disease.

It is beyond the competence of the present study to comment upon the mode of action of mineral-oil adjuvants but the delayed response, shown with influenza B virus, the maintenance of high titres of antibody to a greater extent than two doses of aqueous vaccine and the forcing of antibody levels above the "ceiling" determined by aqueous vaccine might argue in favour of an action of more general significance than the local retention of antigen. The work of Neeper and Seastone (1963) showing that an inoculation of adjuvant, without any specific antigen incorporated, would restore the ability of immunologically paralysed mice to produce antibody would support this. However, from the results with Sendai virus, and others, there is no indication of a general non-specific effect upon antibody forming cells.

There was, likewise, no evidence that adjuvant vaccine elicited antibody of a substantially broader specificity than the aqueous vaccine nor was there a superior capacity to recall antibody to past, and only distantly related, antigens.

THE USE OF INFLUENZA VACCINES.

The protection of the individual and the protection of the community are problems that are related but not identical and thus the solutions adopted may not be the same.

The recent article of Langmuir et al., (1964) once more emphasizes the vunerable position of the elderly and the chronic invalid during an influenza epidemic. The burden of mortality falls on these people and all recommendations for vaccination stress the priority to be given to these groups. Langmuir et al., (1964) feel that even the use of 40 million doses of vaccine had little effect upon the excess mortality associated with the epidemic of 1962-1963 but, considerations of vaccine efficacy apart, Davenport (1963) pointed out that only about 16% of those particularly at risk received vaccine. There is no evidence as yet that would indicate that annual vaccination is unnecessary for individuals who require protection.

In the more general population the use of influenza vaccine by industries to prevent disruption of working schedules has its limitations. It has to be appreciated that the vaccine can only prevent influenza and not other respiratory ailments whether labelled "flu" or not and that the results of vaccination are bound to be disappointing

in non-epidemic years. Although the reactions caused by current vaccines did not appear serious they might elicit consumer resistance - particularly in the absence of epidemics. While the development of more bland vaccines might reduce reactions they would not reduce apathy unless an epidemic was confidently predicted (Davenport, 1963).

As the importance of schoolchildren in propagating influenza epidemics appears to have been shown, it is possible that the control of influenza in this group might have benefits to the community out of proportion to the number of individuals immunised. It might, theoretically, be possible to provide a level of immunity which it did not protect, might so reduce virus multiplication and excretion that the clinical case might be a much less potent source of infection. This, however, has not been demonstrated. During outbreaks among old people and a summer outbreak (Forsyth, 1962) the attack rate in the limited community was high but virus isolation, which might be related to virus excretion, was difficult.

Vaccination of children would be limited by the difficulty and, probably, undesirability of annual inoculation. In addition, vaccine reactions in children tend to be severe and antibody production, particularly to new strains, inadequate. These objections might be

overcome by the use of haemagglutinin-fraction vaccines in adjuvant. However, in view of the possible relation of adjuvant vaccines with hypersensitivity states (Becker, Sparks, Feinberg, Patterson, Pruzansky and Feinberg, 1961, Beebe et al., 1964) and the fibrosis shown to follow the vaccine in the present study the indiscriminate use of adjuvant vaccines in children could only be recommended with some reservation.

However, there is a strong indication for this aspect of control of the disease to be studied both in relation to the normal incidence of influenza and to the possibility of the recurrence of a lethal pandemic. For while there is still uncertainty as to the mode of action and the best method of compounding and using influenza vaccine there is even less understanding of the nature and future of the variations of influenza virus.

CHAPTER V

SUMMARY

The literature relating to the development of inactivated influenza vaccines, their efficacy and reactions to these vaccines has been reviewed and a brief survey has been made of the events which stimulated the demand for vaccines and those properties of the disease which complicate the fulfilment of this demand.

Two trials were carried out on volunteers in which the effects of different doses of aqueous influenza vaccine and different adjuvant vaccines were investigated.

The volunteers were examined and questioned to determine the nature and incidence of reactions following the administration of these vaccines. Systemic reactions were not a problem and while local pain and tenderness were common there was little indication that the vaccine caused interference with normal activities. Following the discovery of subcutaneous nodules after adjuvant vaccines these lesions were reproduced in animals in order that they might be examined histologically.

The antibody response of the volunteers to the vaccines was studied by the haemagglutination-inhibition (H.I.) test and it was shown that antibody titres produced by the adjuvant vaccines persisted longer than those elicited by aqueous vaccines. The effect of the vaccines

on antibody to other strains of influenza virus was investigated and the similarity of response of antibodies to A/Netherlands/65/63 and A/Singapore/1/57 was demonstrated. The sensitivity of the H.I. test was checked by neutralization tests on a limited number of sera.

The effect of influenza vaccines on anti-A_1 iso-agglutinins and haemolysins was investigated in Group O and Group B volunteers.

The results and their relation to the efficacy and acceptability of the vaccines were discussed and some comments on the use of influenza vaccines in the light of the present study were made.

Appendix 1. Forms used in vaccine trials.

Form 1. Explanatory note for first trial.

Explanatory Note.

The trial with influenza vaccines.

Influenza vaccines have been used for a number of years particularly in the armed forces and in industry. These vaccines have been steadily improved. Whereas in past years there have been complaints that some people have had minor reactions such as sore arms after receiving the vaccine the manufacturers claim that their newest, purified vaccines do not cause these reactions. The purpose of this trial is to verify this so that we can quote our own experience for large scale trials which we hope to do next year in school-children.

The vaccines will contain killed virus. They are not experimental vaccines, being available under the National Health Service or much used in the U.S. armed forces. The virus for the vaccine is grown in eggs. Despite the purification procedure there may be a little egg material in the vaccine. A very small number of people show an allergy to eggs and cannot eat them. Therefore these people should not take part in the trial. Active asthmatics also may show allergic reactions to many things and they will be excluded.

The placebo will be a harmless substitute for vaccine so that real and imagined reactions can be separated. All those who take part will receive vaccine before the trial finishes.

Testing the blood samples will give us additional information concerning the vaccines.

It is important to study influenza vaccines because:-

When an influenza outbreak occurs there is much disruption of studies in schools and colleges and of industry.

Influenza is a very serious disease in some people - particularly those who have chest or heart trouble.

An epidemic causing a high death-rate, like that of 1918-1919, may come again at any time and we must have the best methods ready for preventing it.

Form 2. Consent form for first trial.

Consent Form.

Will all those volunteers who are over 21 years fill in part 1 and those under 21 ask their parents or guardian to fill in part 2 of the Consent Form. Please fill in your name in the correct space in block letters.

1. Trial with influenza vaccines.

I, , consent to take part in the proposed trial which involves receiving influenza vaccines and/or placebo inoculations and having blood samples taken. I do not have asthma and am not allergic to eggs.

Signed ..... Date .....

2. Trial with influenza vaccines.

I, , consent to my son/daughter/ward, , taking part in the proposed trial which involves receiving influenza vaccines and/or placebo inoculations and having blood samples taken. He/She does not suffer from asthma and is not allergic to eggs.

Signed ..... Date .....

Form 3. Explanatory note for second trial.

What the Influenza Vaccine Trial (1963-1964) is about

Last winter an extensive trial of influenza vaccines was carried out with the co-operation of the medical students of the then 1st - 5th years Much important information concerning the efficacy of and the reactions to the vaccines has been obtained.

Amongst other things, we discovered that one of the components of the vaccine did not seem to give as good protection as we would have liked.

This winter we want to compare an improved vaccine which is now commercially available in the United Kingdom with another vaccine which has been compounded in a slightly different way to improve its storage properties The trial will be done as follows:-

1. Volunteers will be bled from an arm vein and given an injection of influenza vaccine
2. They will be asked what they think of the injection i.e. did it cause any discomfort
3. All volunteers will be bled again, 28 days and 3 months after the inoculation.

Laboratory tests on the blood samples will give an indication as to how well the vaccine is working. If an epidemic occurs we may be able to see how well the vaccine protects those inoculated.

Research with influenza vaccine is essential because:-

1. Influenza epidemics cause great disruption of industry, education and of the armed forces
2. Influenza can be a very serious disease in some elderly or debilitated people.
3. At intervals a very dangerous type of influenza seems to arise. Therefore we want to know the best way to prevent the disease.

Form 4. Consent form for second trial.

INFLUENZA TRIAL 1963-1964

Consent Form

Will all those volunteers who are over 21 years fill in part I and those under 21 ask their parents or guardian to fill in part 2 of the Consent Form. Please fill in your name in the correct space in block letters.

I. Trial with influenza vaccines

I,..... consent to take part in the proposed trial which involves receiving influenza vaccines and having blood samples taken. I do not have asthma and am not allergic to eggs.

Signed; ..... Date .....

2. Trial with influenza vaccines

I, ... consent to my son/daughter/ ward, ... taking part in the proposed trial which involves receiving influenza vaccines and having blood samples taken. He/She does not suffer from asthma and is not allergic to eggs.

Signed: ..... Date .....

Appendix 2 Haemagglutination - Inhibition Tests.

(a) Treatment of sera

All sera to be used in the H.I. test were treated to remove non-specific inhibitors (Hirst, 1942a, Francis et al., 1946, McCrea, 1946, Chu, 1951, Sampaio, 1952). Two methods were used during the present work.

i Cholera filtrate or Receptor-destroying enzyme (Burnet, McCrea and Anderson, 1947):

Ampoules of tested, lyophilized cholera filtrate were obtained commercially (N.V. Philips-Duphar, Amsterdam). The cholera filtrate was reconstituted with 10 ml. of sterile distilled water per ampoule according to the manufacturer's instructions and the solution was dispensed in the required amounts in sterile 3 x ½ inch Kahn tubes. Serum was added to make a 1 in 6 dilution of serum and this was incubated in waterbaths at 37° C. for 18 hours and at 56° C. for 1 hour. Saline was added to bring the inactivated serum to a dilution of 1:10 and the treated sera were stored at -20° C. until used.

ii Trypsin-periodate:

The technique used was derived from the descriptions of Ananthanarayan and Paniker (1960) and Jensen (1961).

To one volume of serum was added ½ volume of a solution of trypsin containing 8 mgs. of trypsin powder (Trypsin 1:250, Difco Laboratories, Detroit) per millilitre in pH 8.0 M /15 Sorenson's phospate buffer (Appendix 5). The serum/trypsin mixture was incubated in a water bath at 56° for 30 minutes. To the cooled solution

3 volumes of M/90 Potassium periodate (G.P.R., Hopkin and Williams, Ltd.) were added and then was allowed to stand at room temperature for an hour before 3 volumes of 1% Glycerol in saline were added. This, in turn, was allowed to stand for 15 minutes before being made up to a final serum dilution of 1 : 10 with saline.

Because of the lesser effect upon antibody (Tyrrell and Horsfall, 1952) Cholera filtrate was used to inactivate all the human sera in the study except one batch which is mentioned in the text.

(b) Red cell suspensions

i Chicken red cells. Blood was drawn from young fowls by cardiac puncture or from a wing vein. The blood was collected into sterile Alsever's solution (Appendix 5) and stored at 4° C. Before use the red cells were washed thrice in saline and finally packed in a graduated tube by centrifugation and made up with saline to a 0.5% suspension (Lepine, 1954).

It was impossible to use the same fowls throughout this work and young fowls, mostly Light Sussex crosses, were obtained at intervals as needed.

Even in Alsever's solution blood was not kept for more than 4 days and it was usually used within 24 hours of being taken.

ii Pigeon red cells. Pigeon blood was collected by sectioning a wing vein and collecting the blood in Alsever's solution. This was stored at 4° C and used within 48 hours. The cells were washed with

saline and made up to a 0.5% suspension as with chicken red cells.

(c) Virus antigen

Seed virus came from a variety of sources.

1/ A/Singapore/1/57. A fully avid strain was supplied by Dr. A.S. Beare from the Central Public Health Laboratory, Colindale.

2/ B/England/939/59. This strain was supplied by Dr. D. Hobson, Evans Medical, Ltd., and was selected as possessing slightly greater avidity from three other similar strains. In addition, this was the strain in the vaccine.

3/ B/Lee (1940)

B/England/10/54

$A/FM_1/1947$

A/PR-8/1934

A/Swine (Shope - 15). These were obtained as lyophilized allantoic fluid from Dr. H.G. Pereira, World Influenza Centre.

4/ B/Taiwan/4/62

A/Netherlands/65/63 were supplied, as early passage allantoic fluid, by Dr. D. Hobson.

5/ Para-influenza 1 (Sendai). A strain adapted to grow in the egg allantoic cavity was supplied as a lyophilized culture by Dr. Marguerite Pereira, Colindale.

Preparation of antigen

Lyophilized ampoules were reconstituted with appropriate volumes of sterile distilled water and all allantoic fluids were passaged at a

dilution of 10^{-3} and with an inoculum of 0.1 ml. in the allantoic cavities of fertile eggs incubated for 11 days. After a further 48 hours of incubation the eggs were chilled to reduce bleeding, the shell over the air space was removed and the allantoic fluid harvested. Fluids were tested for the presence of haemagglutinin with a 0.5% suspension of chicken red cells. Positive fluids were pooled, clarified by light centrifugation and the haemagglutinating titre determined. Only large batches of a sufficiently high titre were used as antigen. To these thiomersal was added to a concentration of 1:10,000 and the allantoic fluid was dispensed in 4-5 ml. amounts and stored at 4° C.

Special precautions were taken to avoid mixing of cultures.

Formalinized antigens

Dr. D. Hobson kindly supplied some concentrated, formalinized batches of A/Singapore/1/57 (Colindale strain), B/England/939/59 and B/Taiwan/4/62. Owing to the high H.A. titre of this material it was possible to complete large sections of the work with a single batch. The behaviour of the formalinized antigens in the test did not differ from that of active antigens.

(d) Apparatus and diluents:

Perspex plates: Standard W.H.O. haemagglutination plates with 80 cavities (10 x 8) were obtained from R.B. Turner, Ltd. or Prestware, Ltd., London.

Automatic pipettes: Automatic pipettes delivering 0.2 ml. were

obtained from R.B. Turner, Ltd. and modified to an accurate measurement in the laboratory workshop. An 8 x 0.4 cms. glass tube was attached to the Luer fitting nozzle by plastic tubing so that the solution pipetted did not enter the barrel of the syringe and so that material could be taken from a Kahn tube.

Cornwall syringes: Cornwall automatic delivery syringes were supplied by F. Froud and Sons, Ltd., London. These were adjusted to deliver 0.2 ml. volumes (Fig. 39).

Diluent: Initially, "physiological" saline (0.85 gms. of sodium chloride per 100 mls.) was used but this was soon supplanted by 0.01 M Phosphate-buffered saline. This was made up and stored in 10 x concentrated form (Appendix 5) and diluted with water as needed. Each batch of the diluted, isotonic, buffer was tested for pH (7.0 - 7.1) and for satisfactory red cell sedimentation qualities before being used. The word "saline" will be used indifferently for the "physiological" and phosphate-buffered solutions. The H.I. titres were not affected by the use of either diluent but red cells tended to go brown in the non-buffered solution.

It was found that the type of water used for preparing the solutions was important. Demineralized water (Permutit Portable "Deminrolit" Plant Mk. 4) in the buffer solution was associated with poor red cell sedimentation. The red cells failed to form a neat, close button when fully settled. This problem was avoided by using water that had been doubly-

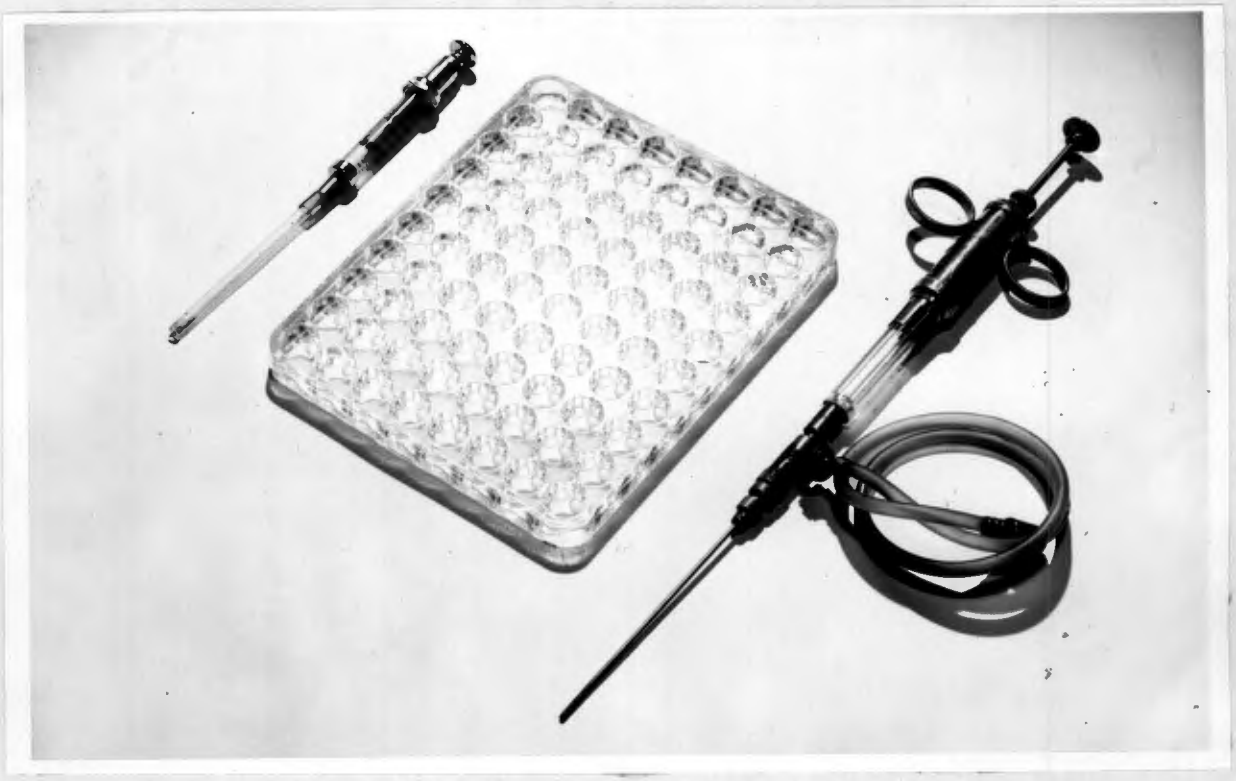


Figure 39. Automatic pipette, haemagglutination plate and Cornwall syringe as used in the H.1. tests.

distilled in glass.

(e) Technique:

Stock virus antigen was titrated by making doubling dilutions with saline from 1:2 - 1:1024 or 1:2048 in a row of cavities in a perspex plate. Volumes of 0.2 ml. were used and pipettes were changed at every dilution. One volume of 0.5% suspension of chicken red cells was added to each dilution and to a control cavity with one volume of saline. The haemagglutination pattern was inspected at the end of an hour when the cells in the control cavity had settled completely. Haemagglutination in each cavity was scored according to the following scheme - taken from the pamphlet on the H.I. test circulated by the World Influenza Centre:-

i ++ Complete agglutination. The agglutinated cells form a granular disc over the bottom of the cavity.

ii ± about 75% agglutination. Here the disc is incomplete and a portion of the cells are not agglutinated.

iii + 50% agglutination. Whereas there is a "halo" of agglutinated cells, a wide ring of non-agglutinated cells is present as well.

iv ± 25% agglutination. A narrow ring of non-agglutinated cells at the bottom of the plate cavity has some clumps of agglutinated cells around it.

v - No agglutination. The red cells settle into a tight discrete button of cells at the bottom of the cavity.

The end-point was read at 50% agglutination and, where necessary, the titre was deduced by interpolation according to the pamphlet mentioned above.

The stock virus was diluted so as to give a sufficient volume of antigen of 8 H.A. unit concentration. Before use the haemagglutinin titre was checked and adjustments and further titrations carried out as required in order to have antigen of the correct strength.

Doubling dilutions of the treated sera were carried out in the perspex plates from 1:10 - 1:5120. 8 sera were titrated per plate. The initial volume of saline for dilution was distributed with a Cornwall syringe. Automatic pipettes were used to mix and to carry the 0.2 ml. volumes of serum dilution from cavity to cavity. At the end of a row the surplus volume of 1:5120 dilution was discarded and the pipette washed by pipetting and discarding clean saline before a new serum was started. As the thawed sera sometimes produced precipitates and often the colour was seen to be in layers it was necessary to mix the sera before sampling.

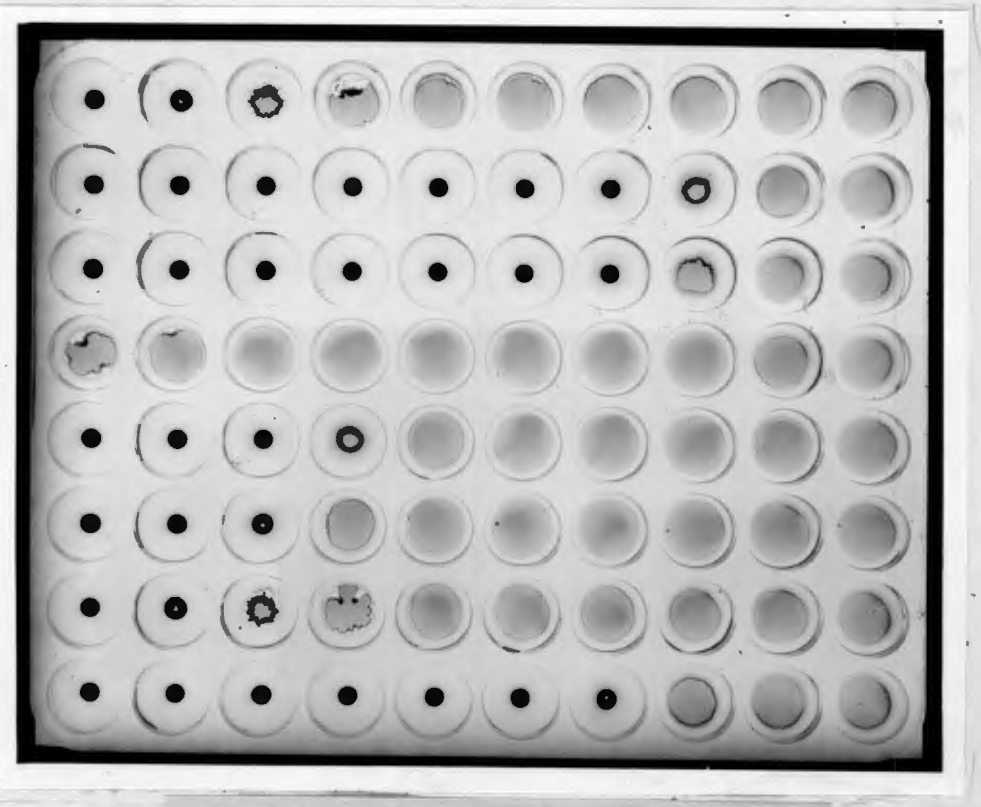
To each volume of serum dilution was added one volume of the appropriate virus antigen and, 20 minutes later, a volume of 0.5% chicken red cell suspension. The 20 minute period was introduced mainly for reasons of convenience. For the early part of the work the antigen was dispensed with automatic pipettes but they were replaced by Cornwall syringes for this purpose. The plates containing the three volumes were allowed to stand at 4° C. until the red cells had settled

satisfactorily - usually 75-90 minutes. Settling at 4°C. was introduced because the temperature of the laboratory frequently rose to 80° Fah. (27.7° C.) or more and as large numbers of sera were dealt with there was need of elasticity in timing.

When ready the plates were read over a translucent pearl glass plate illuminated from below. A hand lens was used to check agglutination when necessary. Cavities were scored from - to ++ as previously indicated and the result entered directly on to special forms. These forms had "blocks" of 8 squares cyclostyled on, representing the perspex plate with its 80 cavities. Against each row of squares the number and letter of the serum was previously entered and against each "block" was the number with which the relevant plate was labelled. (Fig. 40). There was no indication on these forms of the vaccine group of the volunteers from whom the sera came so that the test was performed and read "blind". Titres were calculated from the records later.

Where 50% agglutination was not present the titre was calculated by means of the following scheme - taken from the World Influenza Centre leaflet:

Cavity number			50% end point	Arithmetic reading
2	3	4	Cavity number	(if cavity 2 is 1:20)
-	±	++	$3\frac{1}{4}$	1:50
-	+	++	3	1:40
-	++	++	$2\frac{3}{4}$	1:35
-	++	++	$2\frac{1}{2}$	1:30
-	±	++	$3\frac{1}{2}$	1:60



	10	20	40	80	160	320	640	1280	2560	5120	
195 Y	−	−	+	±	H	H	H	H	H	H	40
Z	−	−	−	−	−	−	±	+	H	H	1250
B	−	−	−	−	−	−	±	±	H	H	960
207 Y	H	H	H	H	H	H	H	H	H	H	<10
Z	−	−	−	±	H	H	H	H	H	H	100
B	−	−	±	H	H	H	H	H	H	H	50
208 Y	−	−	+	H	H	H	H	H	H	H	40
Z	−	−	−	−	−	−	±	H	H	H	800

Figure 40. H.I. plate with system of marking results.

In the presence of serum the settling pattern of the cells varied and in reading the plates allowance had to be made for this factor. The presence of much haemolysis in the serum initially tended to make the tests difficult to read owing to the abnormal red cell patterns at low dilutions. The method of inactivation also influenced this serum effect. It was not possible to use the trypsin-periodate method on rabbit sera because abnormal agglutination obscured the H.I. activity of the serum.

Disposal and cleaning of apparatus:

After being read the perspex plates were immersed in water until the red cells were lysed or washed off. The plates were then transferred to soak for 1 - 2 hours each in normal hydrochloric acid and normal sodium hydroxide successively, with a wash in water between. After soaking in the sodium hydroxide solution the plates were repeatedly washed in running water then soaked in running water for a further 1 - 2 hours or more before receiving a final wash in demineralized water and being dried in the hot room (37° C.). By this means it was possible to use the same plates day after day. To the initial water wash, contaminated with virus and red cells, Chloros Bleach (Imperial Chemical Industries) was added and allowed to act before the water was discarded.

Cornwall syringes and automatic pipettes:

These were washed through with saline, 70% alcohol and water before being stored. Both were frequently stripped and all springs, valves and working surfaces cleaned to maintain them in working order.

Appendix 3:

Preparation of specific immune sera:

Immune sera, to incorporate into tests as standards, were prepared in animals.

Sera to A/Singapore/1/57 and B/Japan/56 were initially prepared in rabbits, as recommended by Lepine (1954), by three, weekly, intraperitoneal inoculations of infected allantoic fluid. The rabbits were bled a week after the last inoculation.

Latterly, sera to all the viruses used were prepared in young fowls by a schedule of inoculation on a single day. An emulsion of infected allantoic fluid and complete Freund's adjuvant (Difco Laboratories) was made by repeatedly sucking and squirting a 1:1 mixture of the two fluids with a syringe. The emulsion was inoculated in 0.25 ml. amounts in both legs and into the breast muscles on both sides. Allantoic fluid without adjuvant was inoculated intraperitoneally. The fowls were bled before and three weeks after inoculation and the sera tested before the birds were bled out.

The bloods were allowed to stand at 37° C. in an incubator for about two hours to clot and then put at 4° C. overnight for clot retraction to take place. The separated sera were stored at -20° C. and a portion was treated to remove inhibitors and was used as required.

Appendix 4:

Neutralization Test

The technique of the neutralization test used was virtually identical with that described by Beare (1962) and McDonald, Zuckerman, Beare and Tyrrell (1962) and was based on the recognition of influenza virus multiplication in monkey kidney tissue culture by haemadsorption (Vogel and Shelokov 1957).

Materials:

Tissue culture:

Monkey kidney tissue cultures were prepared after the method of Melnick (1955). Kidneys were removed from rhesus monkeys under sodium pentobarbitone anaesthesia. The kidney was washed in Hanks' balanced salt solution (Appendix 5) with 0.5% Lactalbumin hydrolysate and antibiotics added, decapsulated and the cortex cut off in small pieces. The medulla was discarded. The fragments of cortical tissue were washed in the Hanks' based solution above to remove excess blood, placed in a conical flask and warmed (37°C.) 0.25% trypsin solution (Appendix 5) added. The mixture was kept in the 37°C. room, stirred with a magnetic stirrer for 30 minutes and the first amount of trypsin discarded. Then trypsin solution was allowed to drip slowly into the bunged flask from a round-bellied funnel in such a way that the increasing pressure thus caused, forced trypsin solution, now containing detached kidney cells in suspension, to rise up a collecting tube which runs from the bottom of the flask through the

bung and flow into a collecting bottle (Fig. 41). The collecting bottle was packed in ice to inhibit further tryptic action. The rate of inflow of fresh trypsin was controlled, by a pinch cock, to allow adequate separation of cells to occur. Stirring continued throughout. The process was allowed to continue until the kidney material was digested free of cells. The collected cell suspension was then gently centrifuged to recover the kidney cells. These were washed free of trypsin and diluted in a growth medium of Hanks' balanced salt solution with 0.5% Lactalbumin hydrolysate, 10.0% ox serum and 0.037% sodium bicarbonate.

The cells from two kidneys were made up to 500 - 700 ml. depending on the yield and the dilute suspension was dispensed into 6 x $\frac{5}{8}$ inch tubes at 0.5 ml. per tube. The tubes, fitted with bungs, were incubated stationary, lying at a gentle tilt, for 4 - 5 days and were then rolled in drums (Malburn, Ltd., London). When the metabolism of the growing cells made the medium acid the tubes were fed with medium 199 (Morgan, Morton and Parker, 1950, Appendix 5) containing 2% calf serum. Tubes were ready for use in 8 days.

An attempt was made to use a continuous diploid line of vervet monkey kidney tissue culture cells. However, haemadsorption was not satisfactory with these cultures and they were not used in the tests.

Virus seeds:

The same strains of A/Singapore/1/57 and B/England/939/59 that were used in H.I. tests were used in neutralization tests. Virus was grown in the allantoic cavities of developing eggs as described above.

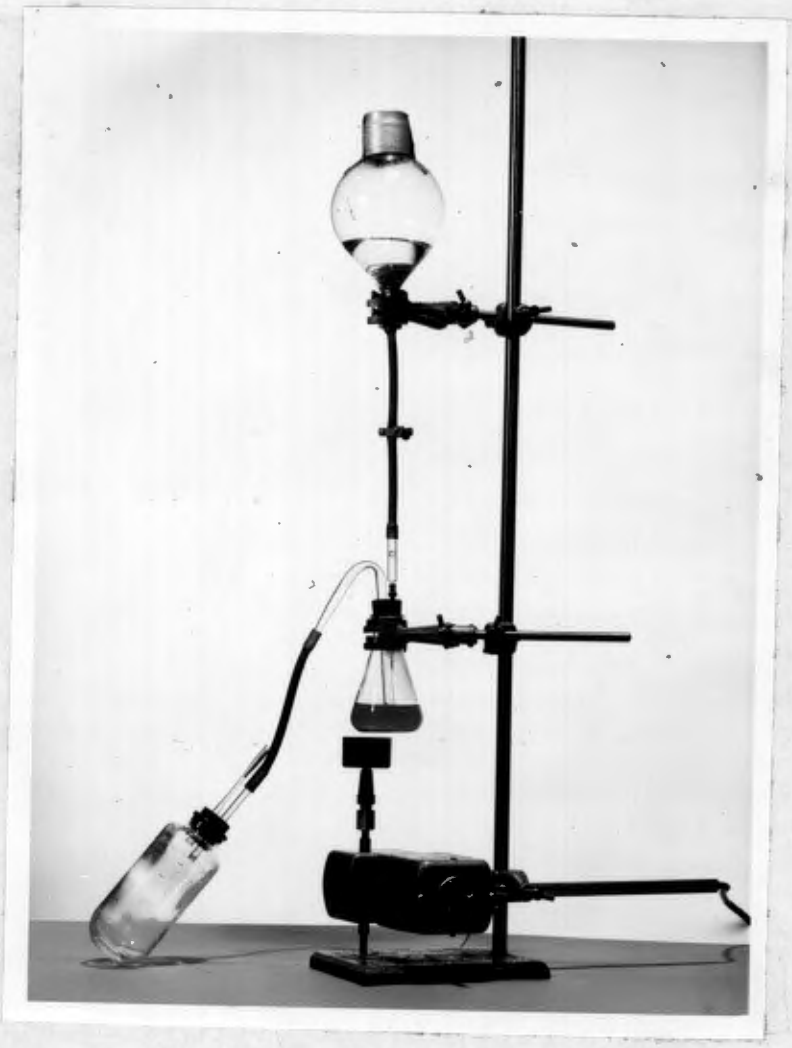


Figure 41. Apparatus for trypsinising monkey kidney. The ice container has been removed to show the collecting bottle.

Batches of clarified infected allantoic fluid with satisfactory H.A. titres were dispensed into labelled soft glass ampoules and these were sealed and snap-frozen in a freezing mixture of dry ice and alcohol and stored in an electrical -70° C. refrigerator (Revco Subzero Equipment). Sample ampoules were rapidly thawed and ten-fold dilutions made in Medium 199. Each dilution was inoculated into four monkey kidney tissue culture tubes. After two days' incubation at 37° C. 0.2 ml. of a 0.4% guinea-pig red cell suspension (see below) was added to each tube and to control, uninoculated tubes. The tubes were sloped at room temperature for 20 minutes so that the red cells could lie in contact with the tissue culture monolayers. Then the tubes were stood upright to allow non-adherent cells to settle and the monolayers were examined microscopically for the characteristic red cell adherence shown in the haemadsorption phenomenon where groups of red cells stick to virus infected cells on the monolayer (Figs. 42 and 43). The end point was determined by the method of Kaerber (Irwin and Cheeseman, 1939) as Haemadsorption doses (H.D.). When repeated tests had shown the titre of a batch of virus to be high, constant and stable it could be used in the tests.

Sera:

All sera to be tested were inactivated at 56° C. for 30 minutes. All the sera of a volunteer were tested at the same time.

Dilutions were made in Medium 199. An initial dilution of 0.5 ml. serum in 2.0 mls. of medium was made followed by doubling dilutions of 2.0 ml. volumes up to the titre required. One pipette

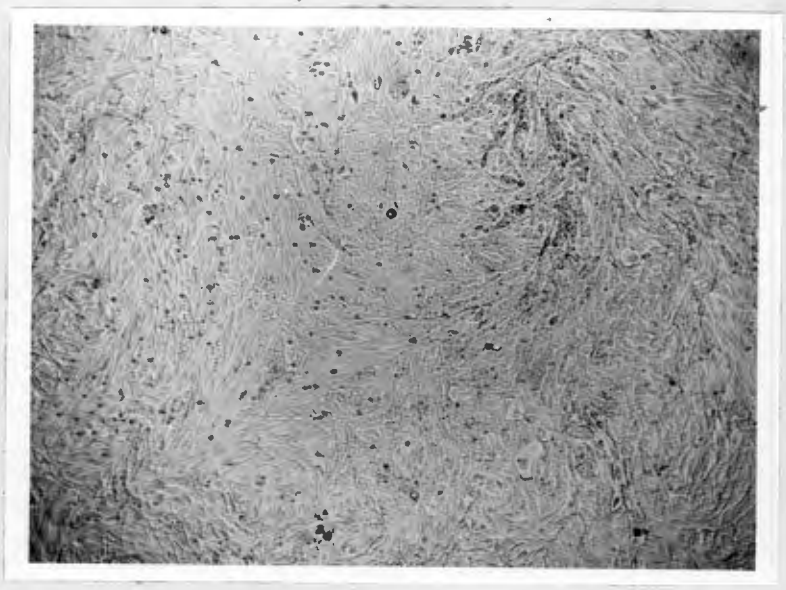


Figure 42. Primary monkey kidney tissue culture, negative haemadsorption. (x 48)

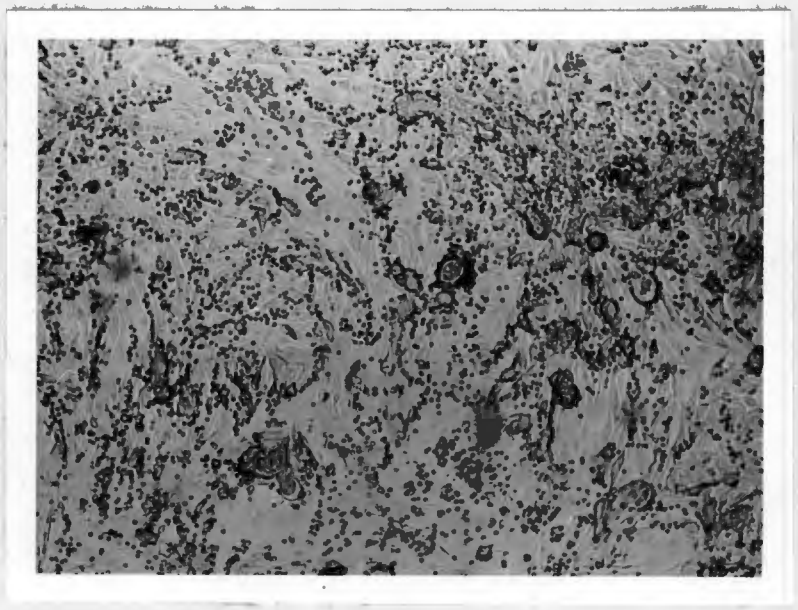


Figure 43. Primary monkey kidney tissue culture, positive haemadsorption. (x 48)

was used per serum. The serum dilutions were dispensed in 3 x ½ inch Kahn tubes so that 1.0 ml. was available for each virus that was to be used at that dilution. The dilutions were performed the day before the test and stored overnight at 4° C.

Guinea-pig cells:

Guinea-pigs, under ether anaesthesia, were bled by heart puncture into Alsever's solution and the blood stored at 4° C. Prior to use the cells were washed three times in saline and made up to 0.4% suspension in saline. It was found essential to use the cells within a day or two of bleeding as cells which were stored for longer than a few days often adsorbed to the tissue cultures in a non-specific manner which, although confusing, could usually be distinguished from haemadsorption caused by the multiplication of influenza virus.

The test:

Ampoules of virus seed were rapidly thawed and diluted in Medium 199 to give 200 H.D. per millilitre. 1.0 ml. of virus was added to each millilitre of serum dilution and the virus-serum mixtures were shaken and allowed to stand at room temperature for an hour. Controls, without serum, were set up with virus at 100 H.D., 10 H.D., 1 H.D. and 0.1 H.D. per ml.

Then 1.0 ml. of each virus-serum mixture and of the controls was inoculated into each of two tissue culture tubes from which the medium had been poured off. The inoculated tubes were rolled at 37° C. for an

hour, the inocula poured off and each tube fed with 2 mls. of Medium 199 and then rolled for 2 days at 37° C.

As feeding the inoculated tubes involved large numbers and as a non-touch technique was essential a Struers (Camlab (Glass) Ltd., Cambridge) electral automatic pipette was adapted for the purpose (Fig. 44). The syringe, valve, inflow and outflow assemblage was autoclaved and connected to the machine just before use. Medium was fed in by gravity and the machine set to dispense 2.0 mls. on the touch of the switch button. No problems with contamination arose with this and trials showed that the tissue cultures were not adversely affected by the medium having passed through the apparatus.

After the inoculated tubes had rolled for two days, 0.2 ml. of 0.4% guinea-pig cells were added to each tube and they were tested and examined for haemadsorption as mentioned above.

The control tubes were read before the tubes inoculated with the serum dilutions. The highest serum dilution with no visible haemadsorption in either of the tubes was taken as the neutralizing titre. From the system of dilution employed only 0.5 ml. of each serum diluted 1:5 was available. This was enough for the inoculation of only one tube and could only be tested against one virus. As, in the neutralization test, the final serum dilution was read this meant that readings at the level of 1:10, unlike other dilutions, were based on one tube only.

Figure 44. Struers electrical automatic pipette adapted to dispense sterile tissue culture maintenance medium.

Appendix 5

Solutions:

The composition or derivation of solutions used in the experimental work, and which have not been specified in the text, are as follows:-

Alsever's solution

Glucose	20.5 g.
Trisodium citrate	8.0 g.
Sodium chloride	4.2 g.
Citric acid	0.55 g.

per Litre.

Phosphate-buffered saline:

a/ 0.01 M pH 7.0

Made up as 10 x concentrated solution and diluted before use.

NaCl	85 g.
Na_2HPO_4	10.61 g.
$Na\ H_2\ PO_4\ 2H_2O$	3.89 g. per Litre

b/ Sorenson's M/15

Stock solution A $KH_2\ PO_4$ 9.08 g./Litre

Stock solution B $No_2\ HPO_4\ 2H_2O$ 11.88 g./Litre

For pH 8.0 mix A - 5%, B - 95%

For pH 7.3 mix A - 20%, B - 80%

Hanks' Balanced Salt Solution:

Containing	NaCl	8.0 g.	
	KCl	0.4 g.	
	$MgSO_4 . 7H_2O$	0.1 g.	
	$CaCl_2$	0.14 g.	
	Na_2HPO_4	0.06 g.	
	$K H_2PO_4$	0.06 g.	
	Glucose	1.0 g.	
	Phenol Red	0.01 g.	per Litre

Penicillin and Streptomycin were added to a concentration of 100 units and 100 ug./ml., respectively.

Trypsin solution:

2.5 g. of Trypsin 1:250 Difco (Difco Laboratories)

in 1 Litre of balanced pH 7.5 Phosphate-buffered saline (Dulbecco and Vogt, 1954)

Medium 199:

Obtained commercially from

1/ Glaxo Laboratories, Ltd., Greenford, Middlesex - ready to use.

2/ Burroughs Wellcome and Co., Ltd., London - as a 10 x concentrated solution without bicarbonate or antibiotics. 500 mls. of the concentrate was made up to 5 litres with distilled water and antibiotics added - as to Hanks' solution above. Sodium bicarbonate was added to a concentration of 0.035 g.% and the resulting solution was filtered through a sintesed glass filter and dispensed in 500 ml. amounts. Further sodium bicarbonate was added as required.

Appendix 6

Tests performed in the study of antibodies to Group A substance.

(a) Solutions:

Saline

Saline used in these tests contained 0.85 g. sodium chloride in 100 ml. of distilled water.

For washing pig cells prior to the Antibody-Absorption test a saline solution containing 1.5 g. sodium chloride in 100 ml. water was used.

Acid-citrate-dextrose

This solution was made up after Loutit and Mollison (1943) in the following manner:

Disodium citrate (monohydric)	2g.
Dextrose (anhydrous)	3g.
Water	120ml.

This volume of water was added to each standard 540 ml. blood transfusion bottle for receiving donor blood.

Phosphate-buffered saline

For use in papainizing red cells Sorenson's pH 7.3 (Appendix 5) buffer was diluted 1:10 with isotonic saline. This resulting solution is diluted 1 in 10 with isotonic saline (see above) for papainizing red cells.

(b) Erythrocytes used in tests:

1 Human A_1 cells: Group A donor blood was collected into

acid-citrate-dextrose solution and stored at 4° C. The cells were grouped and sub-typed with specific anti-A_1 serum. Before use the cells were washed three times in saline by centrifugation and re-suspension. The cells were finally made into a 5% suspension in saline. The batch of red cells was changed weekly.

Other Human cells, as for grouping, were obtained and prepared in a similar manner.

2. Pig cells: Pig blood was collected at the abattoirs weekly. Blood from each of 18 pigs was added to 7 ml. of 3.4% sodium citrate solution to fill a 30 ml. container. The citrated blood was stored at 4° C.

Pig cells were washed three times in saline and made up to a 5% suspension. The cells were grouped into "AP" and "OP" cells in two stages.

i/ Screening: Cells were tested for haemolysis with a human serum known to have anti-A^P specific lysins. To one volume of serum and one volume of fresh, lysin-free, human Group O serum (as a source of complement) was added one volume of the cells to be tested. The mixture was incubated for an hour at 37° C if the lysin containing human serum was derived from a post-natal case, or at room temperature if the serum came from a pre-natal case, and examined for lysis. Cells showing complete lysis were provisionally described as A^P cells.

ii/ Agglutination with pig sera: Cells provisionally grouped as A^P by the screening technique above had the group confirmed

with pig sera after Faistlough (1963).

The cells to be grouped were papainized (Dunsford and Bowley, 1955). To two volumes of papain solution, containing 1 gram of papain powder per litre of isotonic pH 7.3, M/150 phosphate buffered saline, one volume of packed pig cells were added and incubated at 37° C. for 30 minutes. The cells were then centrifuged from the papain solution and washed three times in saline. One volume of a 5% suspension of papainized cells in saline was added to one volume of serum from a "Group O" pig and, in parallel, to a volume of serum from a "Group A" pig. The mixture was incubated at 37° C. for an hour and examined for agglutination. The "Group O" pig serum strongly agglutinated papainized A^P cells. The "Group A" pig serum acted as a control.

(c) Blood grouping:

The bloods of all volunteers in the trials were grouped using both serum and cells.

i/ Grouping the cells:

A drop of the volunteers' cells suspended in saline was added to each of three round-bottomed 2 x $\frac{3}{8}$ inch agglutination tubes containing a drop of anti-A_1, anti-B and Group O serum respectively. The mixture was left at room temperature for an hour and the tubes examined for agglutination.

Grouping sera are supplied to branches

of the National Blood Transfusion Service by the Medical Research Council Blood Group Reference Laboratory, London.

ii/ Serum grouping:

To a drop of the volunteer's serum in each of three agglutination tubes was added a drop of a suspension of A_1, B, and O cells, respectively. The tubes were examined for agglutination after an hour at room temperature.

These tests were carried out and read separately. Further tests were carried out if there was ambiguity or lack of agreement.

(d) Titration of anti-A_1 iso-agglutinins:

Using a marked Pasteur pipette, one volume of saline was added to each of a row of 10 round-bottomed agglutination tubes except the first. To the first and second tube one volume of the serum to be tested was added. After mixing, one volume from the second tube was transferred to the third for mixing. Thus, doubling dilutions were made from 1:1 to 1:512. The final surplus volume was discarded from the last tube and the pipette washed, by repeatedly pipetting and discarding clean saline, before another serum was started.

To each tube one volume of a 5% suspension of standard A_1 cells (see above) in saline was added. The tubes were shaken and allowed to stand at room temperature for $1\frac{1}{2}$ hours. Tubes were then examined for agglutination and the end point determined microscopically. If titres were read they were taken at the last tube showing agglutination to a degree greater than a microscopic trace.

All the sera from one volunteer were titrated together.

(e) Tests for haemolysins:

Duplicate sets of three round-bottomed agglutination tubes were set up for each serum to be examined. A drop of serum was put into every tube and to these a drop of a 5% suspension in saline of human A_1, O^p or A^p cells was added. One set was incubated at 37° C. and the other at room temperature.

After $1\frac{1}{2}$ hours each set was examined and the degree of haemolysis graded. The grades were expressed in the text as:

No Haemolysis

Trace - slight colouring of the supernatant fluid

Partial - stronger colouring of the supernatant but non-haemolysed cells are always visible

Complete - complete haemolysis

(f) Antibody-Absorption test (Tovey, Lockyer, Blades and Flavell, 1962)

A^p cells were washed three times in 1.5% saline and finally packed by centrifugation at 3,000 r.p.m. for 15 minutes.

The serum to be tested was inactivated at 56° C. for 30 minutes. To one volume of serum was added an equal volume of packed A^p cells. The mixture was allowed to stand for an hour at room temperature and then centrifuged to obtain a clear supernatant fluid for testing.

Doubling dilutions in saline were made of the serum 1) before absorption and 2) after absorption. To each dilution was added a volume of a 5% saline suspension of A_1 cells. After 1 hour at room

temperature the tubes of all dilutions were centrifuged for 1 minute at 1,000 r.p.m. and examined for agglutination.

The result was expressed in terms of a comparison of the anti-A_1 agglutinin titres before and after absorption with AP cells. In a weak positive a difference of 1 or 2 tubes (2 or 4 fold) would be noted, a moderate positive - 3 to 4 tubes, and a strong positive - 5 tubes or more.

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