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GENETIC VARIATION AMONG INFLUENZA VIRUSES

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PREFACE

Influenza is still a major threat to the health of mankind because unlike smallpox, polio, mumps, measles, or rubella it is not yet subject to either an effective prophylaxis or therapy. Year after year influenza inflicts enormous human suffering in the form of high morbidity and increased mortality. In addition, the economic loss resulting from the loss of man hours runs into billions of dollars every year. It is no wonder that influenza has been the subject of intense study by clinicians, virologists, epidemiologists, molecular biologists, immunologists, geneticists, public health officials, and others over the last four decades.

This ICN-UCLA symposium was organized to bring together people from different disciplines working with the common objective of reducing the ravages of influenza and to expose them to the totality of the problem of influenza. The arrangement of the conference provided a setting for both formal (plenary session) and informal (poster session, group discussion) exchange of ideas. The number of participants (approximately 120) was optimum for both formal and informal gatherings without any further subgrouping, and thus enabled the participants to join each session without missing another.

This volume documents the proceedings of this major international conference on genetic variation among influenza viruses held at Salt Lake City, Utah from March 8-13, 1981. It includes papers presented by the speakers of the plenary sessions as well as the keynote speaker, Sir Charles Stuart-Harris. It also includes the selected papers presented in the poster sessions. Also included by popular demand are two poems on influenza by Dr. Edwin Kilbourne, which were presented at the conference banquet. The quality of the papers (including the poems) reflects the high standard of the meeting. As stressed in organizational communication to the speakers, most papers contain information not published before.

The papers presented at the meeting included nearly all major aspects of influenza in which important advances are being made. Because of recombinant DNA technology and rapid DNA sequencing, a number of genes of influenza virus from a number of strains have been either completely or partially sequenced. Among these, the gene coding for hemagglutinin (HA) has been most intensively studied and the HA of one or more strains from each subtype (H^1, H^2, H^3) has been completely sequenced. The question of drift and shift at the genomic level was discussed by a number of speakers (Brownlee, Davis, Fields, and Fiers). It became clear that these nucleotide changes are not limited to HA only but occur among other genes as well (Air, Blok, Palese, and van Wyke). More important, these clones are now being successfully used to express viral proteins in both prokaryotes (Davis) and eukaryotes (Hartman and Lai). It is expected that this area will be the subject of intense study in the next few years because of the biological significance of the expression product of cloned genes either for use in prophylaxis or for studying their function. Also, the complete sequence of a DI RNA was presented, locating the point of deletion in the progenitor (PI) gene (Nayak).

The three-dimensional structure of the hemagglutinin gene which has been recently completed was presented by Ian Wilson (it is unfortunate that this important paper was not submitted for publication in this volume). The topological significance of epitopes and other functional domains of HA molecules which have been proposed by either sequencing proteins, nucleic acids, or using monoclonal antibodies or cyanogen bromide cleaved fragments were presented by Laver, Webster, Gerhard, Sleigh, and Jackson. Interesting discussion followed to correlate a direct structural relationship of a sequence(s) to its proposed function(s).

The role of the capped host mRNA is the process of initiation of transcription was further defined by Krug, and the regulation of viral transcription was discussed by Mahy. Lamb's original observation that the NS RNA segment can code for multiple mRNAs and proteins was extended to the RNA coding for M protein(s) (Lamb and Palese) as well as to the RNA coding for NS proteins of influenza B virus (Lamb). Newer information on the biosythesis of hemagglutinin and nature of carbohydrate determinants was presented by Klenk, Compans, Brown, Basak, and Meier-Ewert.

Viral pathogenesis is a complex process involving interaction between virus and host and possibly other environmental factors. Sugiura and Schulman discussed studies conducted toward defining viral gene clusters involved in virulence. Scholtissek presented his studies on suppressor recombinants and suppressor mutants. Schulze and Carroll attempted to define the host receptors for influenza virus while Small discussed the complex host defense mechanism involved in viral pathogensis. Kilbourne discussed complex processes involved in the adapatation of influenza virus in human population. Occurrence of virus variants in human population and its epidemiological signicance was discussed by Kendal, Cox, and Six.

For influenza virologists, the greatest challenge is the control of influenza using an effective prophylaxis. Couch stressed the significance of serum IgG neutralizing antibodies, while Anders, Ennis, and Stein-Streilein discussed T cell-mediated cytotoxicity and its effectiveness in combating influenza viral infections. Dowdle discussed the limitation of present immune prophylaxis and suggested the potential use of concentrated viral vaccine which may be obtained by using newer technology. Murphy and Maassab proposed a number of newer avenues, including the possible use of host range, cold-adapted and temperature-sensitive mutants as candidates for live virus vaccine(s). Only further studies will reveal whether any of these proposed methods, i.e., concentrated antigens (Dowdle), live virus (Murphy and Maassab), or a combination of both (Kilbourne), will be successful in providing an effective immune prophylaxis against influenza. Finally, whichever method is successful in this war against influenza, a lot more understanding of the strategy that influenza virus uses in nature will be required. As Sir Charles Stuart-Harris states in his keynote address, "The present situation calls for redoubling of efforts in order that the quest may become successful and that future generations may have no more fear of influenza than they will have of smallpox.

Funding to defray a portion of the travel expenses incurred by the invited speakers was generously awarded by NIH Contract Number 263-81-0166 (jointly sponsored by the National Institute of Allergy and Infectious Diseases, Center of Disease Control, Bureau of Biologics, FDA, and Fogarty International Center), Beckman Instruments, Endo Laboratories, Merck Sharp and Dohme, and Parke-Davis.

INFLUENZA VIRAL GENETICS AND THE FUTURE

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ABSTRACT

Knowledge gained upon the genetic mechanisms of the influenza viruses is believed to carry implications for the interpretation of the epidemiology of the disease and for its control. Present problems in understanding the epidemiology and in immunization against influenza are described in the light of this thesis.

INTRODUCTION

It was in 1952 that Wilson Smith wrote,"influenza viruses are plastic organisms undergoing constant changes in structure to produce newer forms with changed antigenic constitution, modified architecture and different biological behaviour". But already, influenza viral genetics was being studied experimentally. Burnet and Bull (1943) investigated the haemagglutinin during early passages of human influenza virus A in embryonated eggs by measuring the relative titre for guinea pig and chicken red blood cells. The change on passage in this relation was called the O-D variation. Using the limiting dilution technique to ensure that cultures were derived from minimal infective doses of virus, Burnet et al (1949) showed that it was thus possible to maintain the original 'O' form with its greater avidity for the guinea-pig than chicken red cells. Conversion to the 'D' or derived form with greater avidity for chick cells on further cultivation was explained genetically as a selective survival of mutants. But the recombination of two strains with different characteristics replicating in the same milieu by Burnet and Lind (1949; 1951) first established genetic studies on a firm basis. Henle and Liu (1951) also showed that multiplicity reactivation of partly inactivated virus, was possible, thus providing a further analogy with bacteriophage (Delbrück and Bailey, 1946; Hershey and Rotman, 1948; Luria, 1952).

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The contribution of genetic recombination, now more correctly described as genetic reassortment, to theoretical and practical considerations has been immense. It is widely accepted that its mechanism lies in the segmented structure of influenza RNA revealed by Duesberg and also Pons and Hirst in 1968. Even before knowledge of this structure however, the prophetic words of Burnet in 1951 must be remembered. He wrote that "as a result of some interaction with cell constituents, the virus particle disaggregates (after entry of virus into the cell) and gives rise to a certain number of genetic units". The current intensive study of the genes of the virus by molecular techniques has afforded ample proof of the truth of these remarks.

Problems in the Epidemiology of Influenza

My role in this Conference is to look at and discuss the impact of genetic research on present day problems of human influenza. Let us start upon the epidemiology of human influenza by examining its periodicity.

The most striking feature of influenza is its irregular periodicity whereby it is apparently absent in some years and a cause of large epidemics in others. When many individual countries in one hemisphere suffer epidemics in a relatively short period of time, the occurrence is called pandemic influenza. Formerly, it was argued (Commission on Acute Respiratory Diseases, 1948), that community experience and the wide age-range of influenza suggested that epidemics first caused infection in those most susceptible in whom it produced an immunity which was relatively short-lived. Prevalences were renewed as numbers of susceptibles including those born since the last epidemic, built up in the community. The theory did not explain why epidemics ceased even when as many as half the susceptible persons in the community had escaped infection. However, it was favoured by the finding that between epidemics localised outbreaks and sporadic cases could be located, thus suggesting that virus was persisting in the community. Latterly, however, the view has prevailed that immunological defence of the community resulting from exposure to the surface antigens, the haemagglutinin and neuraminidase of a particular subtype, is defeated by variation, particularly of the haemagglutinin as a result of antigenic drift. Such antigenic variation was originally described by Magill and Francis (1936) and Smith and Andrewes (1938).

The mechanism of antigenic drift was shown by passage of virus in mice partly protected by inoculating a small amount of antibody or by immunization. Virus variants arose whose antigens did not fit the antibodies and which were selectively favoured (Archetti and Horsfall, 1950; Magill, 1955).

To see how closely antigenic drift and epidemicity coincide the experience of a particular country must be examined over a long period of time. Taking mortality from influenza as the index of virus activity, the experience in England and Wales from 1940 to 1970 is that over a whole period in spite of the ageing population, annual mortality has tended to decline. Yet large numbers of deaths occurred in 1943, 1951, 1957-1958 and 1970. The epidemic in 1943 was caused by HoN1 viruses which, as typified by the Weiss strain (Salk et al, 1944), drifted appreciably from WS, PR8 and the 1937 viruses. Secondly, the 1951 epidemic caused great mortality in Liverpool but relatively much less elsewhere and the period from 1946 to 1956 was otherwise marked by relatively smaller prevalences. The 1951 viruses, however, excited much interest because though they had drifted away from the H1N1 prototype of 1946 and 1947, they exhibited two major variants with differing avidity for red cells and Isaacs and Andrewes (1951) were able to follow their separate geographic spread in Europe.

The appearance of the Asian virus (H2N2) in 1957 represented the first antigenic shift of the influenza virus era with alterations in both the H and N antigens and in consequence a large pandemic spread throughout the world in the unprotected population. In fact, this was the largest epidemic of the whole 40 years and, apart from 1958 to 1960, the years from 1957 to 1961 had recurrent prevalences and mortalities without any significant drift of the antigens of the viruses. The last of the Asian (H2N2) epidemics occurred in 1967-68 but soon paled into insignificance when a new pandemic started in 1968 and lasted until 1971 as the H3 haemagglutinin of the A/Hong Kong/68 virus replaced that of Asian strains. Antigenic drift after 1972 of the H3N2 viruses has been closely watched throughout the world. Figure 1 shows the first ten years of the H3N2 sub-type epidemics in England and Wales. It compares the deaths from influenza, influenzal pneumonia and bronchitis with the monthly number of cases of clinical influenza notified to the Royal College of General Practitioners by a panel of 60 G.P's observing a scattered community of 150,000 persons. This index of influenza was used by Clifford et al (1977) in their study of excess mortality because it

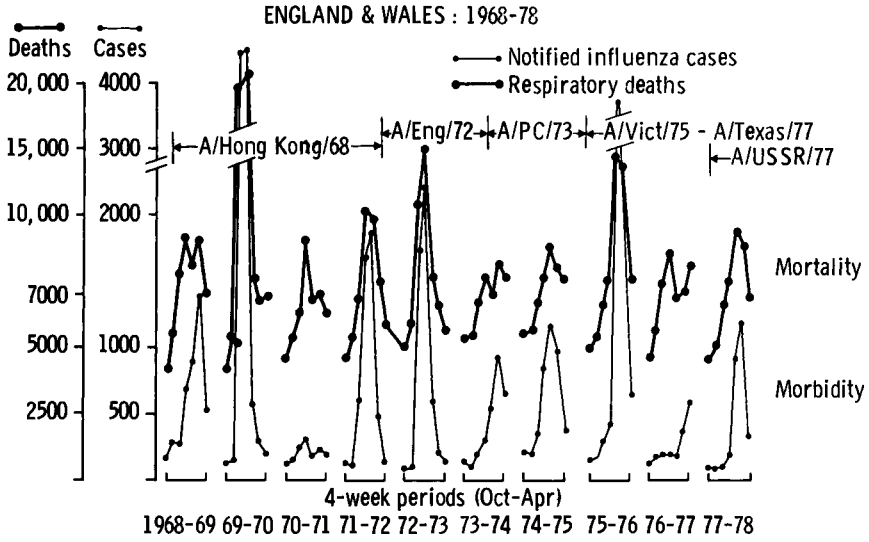

Fig. 1 Antigenic Drift and Influenza Epidemics.
England and Wales, 1968-1978

correlated so well with laboratory evidence of influenza including isolation of viruses. The figure also shows the antigenic variance of the H3N2 viruses up to 1978. The A/England/72 virus appeared briefly at the end of the third wave of A/Hong Kong/68 viruses. It then returned to cause a relatively sharp outbreak in the winter of 1972-1973. Then came the A/Port Chalmers virus which caused relatively feeble outbreaks of 1973-74 and 1974-75. It was succeeded by A/Victoria/75 which everywhere caused a sharp epidemic. This strain was antigenically more remote from A/Hong Kong/68 than its predecessors but the Port Chalmers experience shows that antigenic diversity is not the only factor determining the ability of any particular virus to involve the community. In fact the way in which viral and host factors are intertwined has been well shown by epidemiological experience

since 1977.

The reappearance of the H1N1 subtype of viruses in 1977 in China, then spreading to the USSR and eventually reaching all over the Northern hemisphere was a totally unexpected phenomenon. It is true that a new antigenic shift had been anticipated round about 1978 but reappearance of a former subtype dormant since 1957 was a new experience. The 1977 A/USSR/77 (H1N1) virus was soon recognised to be antigenically close to the 1950-51 viruses (Nakajima et al, 1978; Kendal et al, 1978) which were the variants of the prototype H1N1 virus of 1946-47 (Isaacs and Andrewes, 1951).

The human reaction to the new virus was surprisingly predictable. Serological monitoring had, before the epidemic, shown that persons in the community born since 1957 and aged 20 or less at the time of arrival of the H1N1 virus were without antibodies to this subtype. Infection by H1N1 strains in the USA was confined almost entirely to young adults and children below the age of 26 both in 1977-78 and 1978-79 (Kendal et al, 1979a) and experiences elsewhere were similar. The morbidity and mortality was low in 1977-78 winter but greater in 1978-79 when the epidemic resembled in size the A/Port Chalmers epidemic of 1974-75. Mortality in both these years occurred predominantly in adults particularly in those over 60 and influenza in adults over 25 years of age was produced almost entirely by the continuing prevalence of H3N2 viruses, then the A/Texas/77 variety. What was much more surprising was the escape from H1N1 infection by adults even though these had in many cases relatively low serum HI titres.

Apart from sharp outbreaks of H1N1 influenza from 1977 to 1980 in residential school and other communities, the infection in children and young adults was comparatively mild. This may have been due to the thermal sensitivity (ts) property of the H1N1 viruses found by Oxford et al (1980) which should have rendered the viruses at least partly attenuated. Genetic analysis by oligonucleotide mapping (Young and Palese, 1979) and by competitive RNA hybridization (Bean et al, 1980) has revealed also that the H1N1 viruses recovered in 1978 in California and Brazil, with general circulation in 1979, differed from the A/USSR/77 virus.

Genetic research on antigenic drift and shift

It is uncanny to see the speed with which individual chemical resemblances and differences between the RNA of different strains of influenza virus can now be shown. Recombinant DNA techniques have had considerable success in the elucidation of antigenically-drifted virus. Thus DNA copies

of haemagglutinin genes from viruses of the A/Hong Kong/68 era have revealed nucleotide sequence differences which foreshadow amino-acid differences in the individual haemagglutinins (Verhoeyen et al, 1980), Min Jou et al, 1980). Laver et al,(1980)have also shown alterations of even single amino-acids in the polypeptide sequences of haemagglutinin particularly of the HA1 portion. The picture fits that of a series of point mutations but the precise relationship between amino-acid changes and antigenic variation is so far obscure. What is worse perhaps is the detection of antigenic differences by monoclonal antibodies which appear to have no epidemiological significance (Gerhard and Webster, 1978). Sir Christopher Andrewes drew attention in 1956 to the likelihood that genetic variation would probably throw up differing virus strains, many of which would not be able to spread or even to persist in the partly immune human population. What the clinician and the epidemiologist seek is how to predict the precise antigenic variant which is likely to occur by genetic mutation in the haemagglutinin in the future and which will be epidemiologically successful. This is a tough and perhaps impossible question to answer. Yet without this forecast, the selection of a particular strain or strains of influenza viruses to form the seed for inactivated vaccine for a succeeding year becomes subject to nature's whims and the vaccine may fail to match events. The origin of antigenic shift was formerly explained either on the basis of genetic mutation, now clearly unlikely on grounds of the haemagglutinin structure (Gething et al, 1980), or by persistence of viruses perhaps as a genetic anlage even though inapparent after ten or more years of activity. Reappearance of the H1N1 subtype in 1977 was the first formal evidence that a human virus dormant for 20 years can somehow persist. True, it may have been in a frozen state or it may have been latent in man (Hope-Simpson, 1979) or some other reservoir.

Recycling of former epidemic viruses was first envisaged by Francis et al (1953) and Mulder and Masurel (1958) and the evidence was based on the presence of antibodies to former viruses in sera from persons living during the era when the subtype was prevalent. On this basis also Shope (1944) believed that the A/Swine/31 virus was the survivor in swine of the 1918 virus from man. When the A/Hong Kong/68 (H3N2) virus appeared in 1968 antibodies to its H3 antigen were present in abundance in persons who were alive at the time of the 1890 or earlier pandemics (Masurel, 1969). But this H3 haemagglutinin also had peptide links similar to those of the equine A/Equi 2/63 and duck Ukraine/63 viruses and the meaning of these links was unknown (Laver and Webster, 1973).

Belief in an animal reservoir of human viruses began to be strengthened when it appeared that the A/Hong Kong virus passed relatively rapidly into the animal kingdom and most obviously to domestic pigs after its human debut (Harkness et al 1972).

Latterly the possibility of other animal reservoirs for viruses with human haemagglutinins has been enhanced by work on ducks and other Avian species. Webster et al (1975) showed that antigenic links existed between the Asian virus H2 haemagglutinin and those of two duck viruses isolated in 1972 and 1973 some 4 or 5 years after disappearance of the Asian virus from the human scene. Shortridge (1980) recovered five strains of an H2N2 virus from ducks in Hong Kong in 1978 and in 1979 Shortridge et al found serological evidence in Hong Kong that infection by H1N1 viruses in domestic poultry had occurred in 1975 and 1976, two or three years before the H1N1 virus reappeared in man. Thus, the probability exists that a virus equipped with the genes of former human virus H and N antigens may be sheltering in wild birds and moving from this to man involves the questions 'how' and 'when'. Whether genetic recombination between a human and a duck or other animal virus occurs before the latter can acquire potential for human infectivity and pathogenicity is still unknown. Memory of the failure of the A/New Jersey/76 (Hsw1.N1) virus of Port Dix to establish itself as an epidemic strain is too recent for anyone to think that mere possession of the right surface antigens is the only pre-requisite for a virus to change its host species. However, genetic recombination is a regular phenomenon among the viruses of a single host species such as ducks (Gardner and Shortridge, 1979) and obviously occurs among human viruses. The hunt to uncover the precise mechanism of antigenic shift is clearly on and there could be many surprises ahead.

In the meantime it must be remembered that the goal is not just that of understanding mechanisms but the anticipation of future antigens. Only once has the laboratory permitted the anticipation of a human antigenic variant and that was when Fazekas de St. Groth and Hannoun (1973), derived from A/Hong Kong/68 virus a strain with similarity to the A/England/72 virus which had yet to appear in human infections. But this triumph was short-lived because later variants resembling A/Port Chalmers/73 or A/Victoria/75 did not subsequently appear in the laboratory. Clearly genetic research has a long way to go before it can answer the problem of the source of antigenic shift in the future. However, genetic analysis of the haemagglutinins of animal and human strains should be rewarding, remembering that Scholtissek et al established in 1978 RNA homology between the haemagglutins of Duck Ukraine/

63 virus and A/Hong Kong/68 virus. Also Porter et al (1979) have sequenced the nucleotides of the haemagglutinin gene of fowl plague virus. Knowledge of the sequences of avian and human haemagglutinins though permitting a comparison of structure and of the chemical equivalence of antigens, is still very far from complete. The question remains open as to whether such knowledge will provide evidence of interspecies movement of the genes of antigens such as has been proposed as the basis of antigenic shift. Surely it is necessary also that such a transfer to man of a recombinant from an animal host should be 'caught in the act' before accepting theory as fact. It is a fact that transfer from man to animal kingdom does occur. But when transfer does occur in the reverse direction as for instance of swine influenza virus to man, which occurred sporadically before and after the Fort Dix epidemic of 1976,(Smith et al, 1976; Thompson et al, 1976), the end result may not be a widespread epidemic and the conditions for adaptation to man are not yet understood. The problem of prevention of influenza by immunization. Experiments with influenza vaccines have been conducted for more than 40 years yet a satisfactory basis for their use is still lacking. Inactivated whole virus, split virus or surface antigen preparations are the only varieties available for routine use. The recommendation that immunization should be offered to persons at special risk of dying from influenza because of pre-existing chronic organic disease, still has a shaky foundation. Controlled clinical trials in healthy persons have also given conflicting results dependent in part on diagnostic confusion of influenza with other respiratory virus illnesses. Consequently carefully studied small groups of persons immunized with inactivated vaccine and later challenged with attenuated live virus have been substituted of recent years.

Two major sources of difficulty have become recognised in the use of inactivated vaccine. The first arises from antigenic drift and the realisation that unless the vaccine is produced from a strain of virus antigenically close to that of the circulating virus against which protection is sought, the latter will be relatively less effective even against the comparatively weak challenge of attenuated live virus (Potter et al, 1977). But there is a second problem arising from the fact that the humoral response to inactivated influenza virus is bound by the "original antigenic sin" acquired by the first exposure to the subtype of virus concerned (Francis et al, 1953; Davenport et al, 1953). When antigenic drift occurs, the haemagglutinin of the drifted virus given as vaccine, will in many persons effectively reinforce the antibodies to the prototype of the subtype but may fail to induce a protective level of antibodies to the drifted antigen (Oxford et al, 1979). Cross re-acting heterologous antibody directed against the prototype subtype virus has been shown to be less

protective than that which is specifically directed against the antigen in the vaccine (Couch et al, 1979). Hence even when the closeness of the virus in the vaccine to that in the field has been assured, the vaccine cannot be guaranteed to provide protective efficacy once antigenic drift has occurred.

These twin circumstances of drift and inability to create specific protective antibodies probably explain why inactivated vaccine may fail when given annually to groups of persons exposed to a high risk of infection. Such an experience of inactivated vaccine as that recorded by Hoskins et al (1979) may mean that immunized persons acquire less protection from vaccine than from an attack of influenza by a previous virus of the same subtype antigenically heterologous to the virus causing later challenge. When antigenic shift has occurred, immunization in persons not previously exposed to infection by the new subtype is far less effective serologically than at interpandemic times. Two doses of inactivated vaccine are required and even then antibody levels are lower than after one dose of vaccine in persons who were infected by the same sub-type of virus years before (Nicholson et al, 1979). Nevertheless in spite of all these drawbacks, it has to be realised that inactivated influenza vaccine is still the only immunological weapon licensed and available for use against this unpredictable group of viruses

The picture presented by live attenuated virus vaccine today presents a paradox. On the one hand millions of doses of live vaccine were at one time used in the USSR and in some European countries without apparent harm but with doubtful efficacy. Yet epidemics have continued to occur in the USSR as in other countries so that control of influenza has not been obtained. But in the USA, the UK and Belgium experience in the attempted production of seed virus strains, which are both attenuated and still infective, have been pursued in the past several years with results which are at times encouraging and at other times frustrating. There have been several reviews of these attempts but from the point of view of this Conference, it is important to pinpoint the essential goals and to perceive the role of genetic research in past and future attempts to obtain the ideal attenuated virus for general use as a vaccine.

The desirable properties of the ideal seed virus for such a vaccine were spelt out by Murphy et al in 1976. Of these the essential requirements are attenuation for seronegative persons with retention of infectivity, antibody formation and resistance to challenge infection. But of equal importance is the retention of attenuation by virus discharged to the environment by the vaccinated person and this implies genetic stability of the vaccine virus. The genetic basis of the attenuation of virulence is now recog-

nised as polygenic and this holds for fowl plague virus (Rott et al, 1979; Rott, 1980), for the human ts viruses of Chanock and the cold-adapted viruses of Maassab. The gene segments which contribute to attenuation differ in the two groups of human viruses (Murphy et al, 1980 (a); Massicot et al, 1980; Kendal et al, 1979 (b)). Genetic instability is an apparent weakness of ts viruses when used in wholly sero-negative children and the possible mechanism of this has been described by Murphy et al (1980) (b). The search for a more stable attenuated parent by genetic engineering is in progress (Chanock and Murphy, 1980). The sequencing of RNA segments (Lai et al, 1980), the magic of restriction enzymes used on DNA copies of the RNA and much hope are all thrown into the quest which is of such great importance. But there is no magic concerning the future requirements for live virus vaccines. As in the case of inactivated vaccines progress with live vaccines also depends upon the monitoring of the dance of the H and N antigens of the human viruses throughout the world. Also periodic change in the composition of the vaccines is required and it is this above everything else that suggests a future emphasis upon or at least a parallel role for chemotherapy rather than for vaccines. The pursuit of antiviral chemotherapy. In spite of many attempts to produce compounds which selectively inhibit influenza virus replication in experimental systems without provoking harmful effects on the cells and organs of mammalian hosts, few candidates have emerged. Of those with activity in infected animals fewer still have been worthy of test in man and the results in human influenza have sometimes conflicted with those in experimental studies. Amantadine, 1-adamantanamine hydrochloride, has been endorsed after many trials, for wider use in man. Its chemoprophylactic power against influenza A viruses, irrespective of antigenic subtype, is greater than its therapeutic action once illness has begun. Even so its usefulness in treatment is measurable and required greater exploitation in complicated illnesses. Absence of action against influenza B is a major handicap, however, and may be one reason for the cautious attitude of clinicians.

The recent progress in nucleic acid chemistry has been an enormous stimulus to the discovery of inhibitory antiviral compounds. Nucleosides and nucleotides whose sequences may mimic strategic sequences of viral genes or which can actively interfere with key viral enzymes have become known. One such, the nucleotide Ribavirin or Virazole (1-B-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is active against both influenza virus A and B in vitro (Huffman et al, 1973) in mice (Khare et al, 1973) and ferrets infected with influenza virus (Schofield et al, 1977). In man negative results outweigh the occasional success and the controlled trial by Smith et al (1980) is young adults ill

with influenza due to A/Brazil/78 (HlNl) showed no diminution in symptoms or fever or the presence of virus in nasopharyngeal secretions after oral Ribavirin. This is a toxic compound and its negative result in man may be due to the difference in host metabolism in man compared with that in mice and ferrets.

Other attempts have been made to interrupt viral nucleic acid replication. Oxford and Perrin (1977) have found a number of compounds active against influenza RNA transcriptase. Zamecnik and Stephenson (1978) found a tridecamer polynucleotide with activity against Rous sarcoma virus and the polynucleotides certainly offer antiviral possibilities as indicated by Stebbing (1979) in his extensive review. But inhibitory effects are not limited to the bases as shown by the antiviral action of oligopeptides with aminoacid sequences resembling the N-termini of viral polypeptides and described by Dr. Choppin in this Conference. It is impossible not to believe that the pursuit for successful therapy by rational means will not lead to a blind alley and it is important that no clues should be ignored. The human need for help is very great and the control of influenza by specific vaccines is so hedged around with difficulties due to genetic virus variation that attempts to develop chemical antagonists are of enormous importance. Conclusion. This survey has been an attempt to perceive the contribution thus far made by knowledge of the biological and biochemical mechanisms of influenza virus towards solution of the existing problems in the attempted prevention and treatment of human influenza. It is apparent that immunization has often failed because of inability to perceive the direction of genetic variation in the human viruses of the antigens which determine future epidemics and pandemics. Biochemical research has now begun to discover the chemical basis of antigenic variation in the glycoproteins but it is too early for an improvement in the forecast of future mutational changes in the viruses of next year or the year after. There is evidence of the potential for changes in base sequences and aminoacid translations of viral RNA, some apparently irrelevant and some of antigenic significance. It is the latter which are of epidemiological significance and perhaps an experimental approach is now needed to change the emphasis from past experience to future possibilities.

As I look back however, on the past forty and more years I am impressed by the panorama of the laboratory and the world concept of influenza which has been unfolded in my medical lifetime. Many gaps in knowledge have been successively filled. When the first successful experiments on protection by influenza vaccine were made by the late Thomas Francis and the Commission on Influenza (1944), he was firmly of the opinion that the possible number of antigenic sub-

types of influenza virus A was limited. The alternative view of almost limitless variation was too awful to comprehend. Which view is correct? In my judgement we still do not know. Perhaps our colleagues of the animal influenza field may now understand our concern over the discovery of the many haemagglutinins existing among the avian viruses, if indeed birds are the reservoir of the genes of future human viruses. The message surely to the laboratory is that it is necessary to press on with the genetic, chemical and immunological analysis of the surface antigens of these viruses. As the surveillance of human experiences is still lamentably incomplete, these studies must continue to be regarded as of equal importance.

If I were a young man about to enter the field of virus research, I would take heart from the failure of previous generations. Influenza virus and human influenza continue to present a challenge to the inquisitive and to frustrate clinicians and epidemiologists. Moreover, the threat of the unexpected has not been lifted from those of us concerned with the control of infection by this unpredictable virus. The present situation calls for a redoubling of efforts in order that the quest may be successful and that future generations may have no more fear of influenza than they will have of smallpox.

References

Andrewes, C. H., Calif. Med., 1956, **84**, 375

Archetti, I. and Horsfall, F. L. Jr., J.exp. Med. 1950, **92**, 441

Bean, W. J. Jr., Cox, N. J. and Kendal, A. P., Nature, 1980, **284**, 638

Burnet, F. M., Bull. Johns. Hopk. Hosp., 1951, **88**, 137

Burnet, F. M. and Bull, D. R., Austral. J. exp. Biol. Med. Sci. 1943, **21**, 55

Burnet, F. M. and Lind, P. E., Austral. J. of Science, 1949, **12**, 109

Burnet, F. M. and Lind, P. E., J. Gen. Microbiol., 1951, **5**, 59

Burnet, F. M., Stone, J. D., Isaacs, A. and Edney, M., Brit. J. exp. Path., 1949, **30**, 419

Chanock, R. M. and Murphy, B. R., Rev. Inf. Dis., 1980, 2,421
Clifford, R. E., Smith, J. W. G., Tillett, H. E. and Wherry, P. J., Int. J. Epidem., 1977, 6, 115
Commission on Acute Respiratory Diseases., Amer. J. Hyg., 1946, 43, 29
Commission on Influenza., J. Amer. Med. Assn., 1944, 124,982
Couch, R. B., Webster, R. G., Kasel, J. A. and Cate, T. R., J. Inf. Dis., 1979, 140, 553
Davenport, F. M., Hennessy, A. V. and Francis, T. Jr., J. exp Med., 1953, 98, 641
Delbrück, M. and Bailey, W. T. Jr., Cold Spring Harbour Symposium in Quant. Biol., 1946, 11, 33
Duesberg, P. H., Proc. Nat. Acad. of Sci., 1968, 59, 930
Fazekas de St. Groth, S. and Hannoun, C., Compt. rendu. Acad. Sci., Paris, 1973, 276, Série D, 1917
Francis, T. Jr., Davenport, F. M. and Hennessey, A. V., Trans. Assoc. Amer. Physns, 1953, 66, 231
Gardner, J. D. and Shortridge, K. F., Rev. Inf. Dis., 1979, 1 885
Gerhard, W. and Webster, R. G., J. exp. Med., 1978, 148, 383
Gething, M. J., Bye, J., Skehel, J. and Waterfield, M., Nature, 1980, 287, 301
Harkness, J. W., Schild, G. C., Lamont, P. H. and Brand, C. M., Bull World Health Org., 1972, 46, 709
Henle, W. and Liu, O. C., J. exp. Med., 1951, 94, 305
Hershey, A. D. and Rotman, R., Proc. Nat. Acad, Sci., 1948, 34, 89
Hope-Simpson, R. E., J. Hyg. (Camb.), 1979, 83, 11
Hoskins, T. W., Davies, J. R., Smith, A. J., Miller, C. L. and Allchin, A., Lancet, 1979, 1, 33
Huffman, J. H., Sidwell, R. W. and Khare, G. P., Antimicrob. Agents and Chemoth., 1973, 3, 235
Isaacs, A. andAndrewes, C. H., Brit, med. J., 1951, 2, 921
Kendal, A. P., Noble, G. R., Skehel, J.J. and Dowdle, W. R., Virology, 1978, 89, 632
Kendal, A. P., Joseph, J. M., Kobayashi, G., Nelson, D., Reyes, C. R., Ross, M. R., Sarandria, J. L., White, R., Woodall, D. F., Noble, G. R. and Dowdle, W. R., Amer. J. of Epidem., 1979 (a), 110, 449
Kendal, A. P., Cox, N. J., Galphin, J. C. and Maassab, H. F., J. gen. Virol., 1979b, 44, 443
Khare, G. P., Sidwell, R. W., Witkowski, J. T., Simon, L. N. and Robins, R. K., Antimicrob. Agents and Chemoth., 1973, 3, 517
Lai, C-J., Markoff, L. J., Zimmerman, S., Cohen, B., Berndt, J. A. and Chanock, R. M., Proc. Nat. Acad. Sci., 1980, 77, 210

Laver, W. G. and Webster, R. G., Virology, 1973, 51, 383
Laver, W. G., Air, G. M., Dopheide, T. A. and Ward, C. W., Nature, 1980, 283, 454
Luria, S. E., J. Cell. Comp. Physiol., 1952, 39, Suppt. 1, 119
Magill, T. P., J. exp. Med., 1955, 102, 279
Magill, T. P. and Francis, T. Jr., Proc. Soc. exp. Biol. and Med., 1936, 35, 463
Massicot, J. G., Murphy, B. R., Van Wyke, K., Huang, K.Y. and Chanock, R. M., Virology, 1980, 106, 187
Masurel, N., Lancet, 1969, 1, 907
Min Jou, W., Verhoeyen, M., Devos, R., Saman, E., Fang, R., Huylebroeck, D., Fiers, W., Threlfall, G., Barber, C., Carey, N. and Emtage, S., Cell, 1980, 19, 683
Mulder, J. and Masurel, N., Lancet, 1958, 1, 810
Murphy, B. R., Richman, D. D., Spring, S. B. and Chanock, R. M., Postgrad. Med. J., 1976, 52, 381
Murphy, B. R., Markoff, L. J., Chanock, R. M., Spring, S. B., Maassab, H. F., Kendal, A. P., Cox, N. J., Levine, M. M., Douglas, R. G. Jr., Betts, R. F., Couch, R. B. and Cate, T. R. Jr., Phil. Trans. R. Soc. (Lond.) Series B, 1980a 288, 401
Murphy, B. R., Tolphin, M. D., Massicot, J.G., Kim, H-Y, Parrott, R. H. and Chanock, R. M., Ann. N.Y. Acad. Sci., 1980(b), 354, 172
Nakajima, K., Desselberger, U. and Palese, P., Nature, 1978, 274, 334
Nicholson, K. G., Tyrrell, D. A. J., Harrison, P., Potter, C. W., Jennings, R., Clark, A., Schild, G. C., Wood, J. M., Yetts, R., Seagroatt, V., Huggins, A. and Anderson, S. G., J. Biol. Stand, 1979, 7, 123
Oxford, J. S. and Perrin, D. D., Ann. N.Y. Acad. Sci., 1977, 284, 613
Oxford, J. S., Schild, G. C., Potter, C. W. and Jennings, R., J. Hyg. (Camb.), 1979, 82, 51
Oxford , J. S., Corcoran, T. and Schild, G. C., J. gen. Virol 1980, 48, 383
Pons, M. W. and Hirst, G. K., Virology, 1968, 35, 182
Porter, A. G., Barber, C., Carey, N. H., Hallewell, R. A., Threlfall, G. and Emtage, J. S., Nature, 1979, 282, 471
Potter, C. W., Jennings, R., Nicholson, K., Tyrrell, D. A. J. and Dickinson, K. G., J. Hyg. (Camb.), 1977, 79, 321
Rott, R., Phil. Trans. R. Soc. (London) Series B, 1980, 288, 393
Rott, R., Orlich, M. and Scholtissek, C., J. gen. Virol., 1979, 44, 471

Salk, J. E., Menke, W. J. and Francis, T. Jr., J. Amer. Med. Assn., 1944, 124, 93
Schofield, K. P., Potter, C. W., Phair, J. P., Oxford, J. S. and Jennings, R., Effect of ribavirin on influenza virus infection in ferrets. In Chemotherapy. Ed. J. D. Williams and A. M. Geddes, 1975, Vol. 6, 253, Plenum Press
Scholtissek, C., Rohde, W., Von Hoyningen, V. and Rott, R., Virology, 1978, 87, 13
Shope, R. E., Medicine, 1944, 23, 415
Shortridge, K. F., Res. in Vet. Sci., 1980, 28, 296
Shortridge, K. F., Webster, R. G., Kam, S. L. and Gardner, J. M., Bull W.H.O., 1979, 57, 475
Smith, W., Lancet, 1952, 1, 885
Smith, W. and Andrewes, C. H., Brit. J. exp. Path, 1938, 19, 293
Smith, C. B., Charette, R. P., Fox, J. P., Cooney, M. K. and Hall, C. E., J. Inf. Dis., 1980, 141, 548
Smith, T. F., Burgert, E. O. Jr., Dowdle, W. R., Noble, G. R. Campbell, R. J. and Van Scoy, R. E., New Engl. J. of Med., 1976, 294, 708
Stebbing, N., Pharmac. & Therap. Part A, 1979, 6, 291
Thompson, R. L., Sande, M. A., Wenzel, R. P., Hoke, C. H. Jr. and Gwaltney, J. M. Jr., New Engl. J. of Med., 1976, 295, 714
Verhoeyen, M., Fang, R., Min Jou, W., Devos, R., Huylebroeck, D., Saman, E. and Fiers, W., Nature, 1980, 286, 771
Webster, R. G., Laver, W. G. and Tumova, B., Virology, 1975, 67, 534
Young, J. F. and Palese, P., Proc. Nat. Acad. Sci., 1979, 76, 6547
Zamecnik, P. C. and Stephenson, M. L., Proc. Nat. Acad. Sci. 1978, 75, 280

DRIFT AND SHIFT OF INFLUENZA VIRUS STUDIED AT THE GENOMIC LEVEL

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I. ABSTRACT

Comparison of the hemagglutinin of A/Victoria/3/75 with that of A/Aichi/2/68 shows that all changes are due to nucleotide substitutions, except for one triplet insertion. Substitutions are mostly transitions, possibly due to G:U mispairing during replication. Most mutations causing an amino acid change are selected away, but this selection is less stringent in the HA1 part, expecially in the middle region. Indeed at a

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number of sites the mutation avoids immunological neutralization and therefore survives in the population. The net result is an apparently higher variability in HA1. Of the 22 amino acid changes between the HA1 of Aichi/68 and of Victoria/75, we estimated that 16 were involved in antigenic drift. Recent data of Wiley et al. suggest indeed that 15 of the mutations are in immunologically important positions (5 of which are uncertain) divided over four antigenic domains. Remarkably, the implantation of carbohydrate chains is not completely conserved in these H3 hemagglutinins.

In order to gain information on the origin of the H3 subtype we have now completely sequenced the hemagglutinin gene of A/Duck/Ukraine/1963. Compared to Aichi/68 there are no deletions or insertions, but only 9.1% nucleotide changes and 4.2% amino acid changes. Taking the silent changes as an internal clock and assuming constant mutation rate independent of host, our data suggest that both originated from a common ancestor gene some 9-11 years ago, e.g., around 1949-1953. This recent divergence strongly suggests an animal hemagglutinin gene as the progenitor of the human H3 pandemic. The evolution 1953→1968 was mostly in the absence of strong immunological selection, as the relative number of amino acid changes in the antigenically important positions is less than observed for the comparison Aichi/68-Victoria/75. Also the antigenic domains C and D are conserved between Duck/63 and Aichi/68. This clearly closer homology between Duck/Ukraine/63 and Aichi/68 compared to Duck/63 and Victoria/75 constitutes virtual proof that the 1968 pandemic originated by a recombination event in which an H3 type hemagglutinin gene from an animal reservoir was introduced into a human influenza virus.

II. INTRODUCTION

Genetic engineering methods have led to a tremendous increase in our capability to study genetic material and genetic events. In 1976 it became possible to extend these approaches to the study of RNAs, as Maniatis and coworkers showed that messenger RNA could be faithfully copied into a double-stranded DNA molecule, which was then propagated by cloning it into a plasmid. The reverse transcription reaction required the addition of an oligo(dT)-primer which hybridized readily to the poly(A)-tail of the eukaryotic mRNA. At that time we were studying Bacteriophage MS2 RNA, which was the first viral genome to be completely sequenced (1). Obviously cloning of this genome would open new avenues of further study,

e.g., to elucidate the interdependent regulation of the expression of the viral genes. For this purpose, we worked out a procedure which allowed to add poly(A)-tails to the viral RNA, without any internal nicking (2), and this subsequently allowed the reverse transcription (3) and the cloning of nearly full-length viral RNA (4).

Once this methodology became available, it was obvious to apply it to the molecular study of influenza. Indeed the wide occurrence of this human and animal viral disease, and the remarkable antigenic plasticity of this virus, made it a prime-choice study subject for the new type of molecular biology. As detailed in other contributions of this book, there are two antigenic glycoproteins on the surface of influenza virus, the hemagglutinin (HA) and the neuraminidase (NA). So far, neutralizing antibodies were always found to be directed against the HA. Mainly by serological tests and by protein chemistry analysis two types of changes have been distinguished: Drift is more a gradual change which leads from one epidemic to the next; Shift is a much more drastic change, which usually results in worldwide pandemics as residual immunity in the population is virtually non-existent. The last major shift occurred in 1968 when the Hong Kong flu started to spread all over the world.

Influenza is a myxovirus, and the genome of these is negative-stranded and segmented. Each virion contains eight RNA molecules which can be separated according to size. Each RNA contains negative information; inside the cell it is copied to make a full-size replica which functions in replication, and further copied by a special mechanism to generate positive-stranded mRNAs. Except for the smallest gene, which codes for two non-structural proteins, all other RNAs correspond to one gene, which means they code for one protein. The fourth largest RNA codes for HA and the fifth or sixth – depending on the strain – codes for the NA. As we wanted to understand the molecular basis of shift and drift, we cloned the HA gene and analyzed its nucleotide sequence.

III. THE H3 INFLUENZA HEMAGGLUTININS: DRIFT

We first cloned the HA gene of the strain A/Victoria/3/75 (H3N2) and determined its nucleotide sequence (5). The RNA molecule is 1768 nucleotides long. In this paper we shall discuss the information on the basis of the positive strand, which means the complement of the RNA found in the virus. At the 5'-end there is a 29 nucleotide-long untranslated sequence, followed by the initiation codon AUG. The coding part contains information for a 567 amino acids long polypeptide, followed by

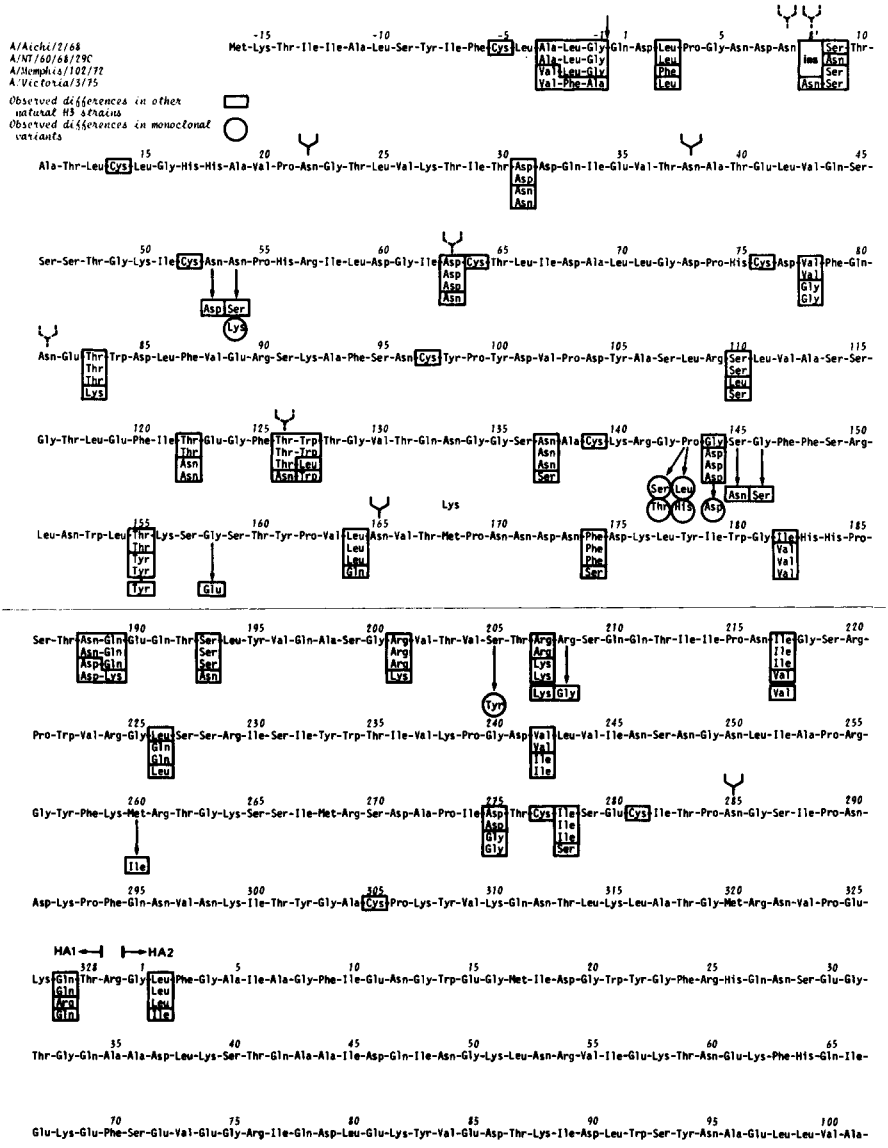
the termination codon UGA, and in phase, a few triplets further UAG.UAA. The untranslated part at the 3' end, including the termination codon, is 35 nucleotides long. The polypeptide starts with a 16 amino acids long signal peptide, undoubtedly involved in secretion of the protein through the membrane. The HA1 part consists of 329 amino acids; it is known that the "head" of the spike is formed by HA1, and that the immunogenic sites are in this part of the molecule. The connection to HA2 is only by a single arginine residue, which is removed upon maturation of the virus. The HA2 chain extends over 221 amino acids. The amino-terminal sequence is highly conserved and remarkably hydrophobic, and could possibly be involved in fusion between the virus and the target cell (6). There is another highly hydrophobic region near the end of the HA2 molecule, from residue 185 to 210; this segment undoubtedly spans the lipid bilayer. It contains a few serine residues, but these could possibly function as anchors for fatty acid attachment (7). The end of the transmembrane segment may be indicated by a basic residue. A short tail of only 11 residues would then be located on the inside of the viral envelope and may possibly interact with the matrix or even the nucleoprotein complex.

Out next step was to clone and sequence the HA gene of the A/Aichi/2/68 strain, a virus isolated at the very beginning of the H3 period (8). This allowed us to analyze the drift which had occurred over the seven year period from 1968 to 1975. Moreover, similar work had gone on elsewhere leading to the elucidation of the sequence of the HA of A/Memphis/102/72 (ref. 9) and of A/NT/60/68/29C (ref. 10). Also the nucleotide sequence analysis by Gething et al. (11) and the protein chemistry analysis by Ward and Dopheide (12,13) should be mentioned in this context. The results of the comparative analyses of the H3 hemagglutinins are summarized in Fig. 1. It may be noted that amino acid changes in the signal sequence are relatively frequent. Nearly all mutations are single nucleotide substitutions, but there is one exception: the Victoria/68 HA has acquired an additional amino acid not present in any other strain. This expansion of the genome could have arisen by slippage of the RNA polymerase; an AACA sequence has become AACAACA. Most of the amino acid changes correspond to a single nucleotide change and often are of a conservative nature. But there is one exception, namely residue 155 of the HA1, which is threonine (ACC) in Aichi/68 and tyrosine (UAC) in Victoria/75. The most remarkable result is that the relative number of amino acid changes is appreciably higher in the HA1 part compared to the HA2 part (6.7% versus 1.8%). The reason for this is obvious: the HA2 part may exert an almost enzyme-like activity and the structure-

function relation imposes such constraints that most mutations leading to amino acid changes are selected away. On the other hand, a number of amino acid positions in HA1 form part of an antigenic site, and if mutated, they allow to overcome the immunity in the population and therefore these mutants acquire a selective advantage. It follows that there is no reason whatsoever to believe that the mutation frequency per se is high in HA1 or in the HA gene. In fact there is even no reason to believe that the intrinsic error-frequency upon replication of influenza virus is higher than that upon replication of, for example, poliomyelitis virus or even of the Bacteriophage MS2. What is different is the extent of elimination of unfavorable mutants. Apparently most of the residues in HA1 which form part of sites towards which neutralizing antibodies can be raised are capable of variation (drift) without affecting the functionality of the molecule. That there is a higher selection pressure in the HA2 part is further proven by considering the number of non-silent to silent mutations. As most of the non-silent mutations are selected away in HA2, the ratio of the former to the latter will be lower in this part of the molecule; indeed the ratio non-silent to silent mutation is 1.12 for HA1 but only 0.45 for HA2.

Most of the mutations can be explained simply by G:U mismatching during replication. Indeed, this would lead to transitions, which are in fact 3.3 times more frequent than transversions.

Twenty-two amino acid changes have occurred in the HA1 part between 1967 and 1975. A number of these undoubtedly are part of antigenic determinants and are directly responsible for the immunological drift. A crude estimate of the number of residues involved can be made taking the mutation frequency observed in HA2 as a background. After correction for the difference in length, a value of about 16 excess amino acid changes in HA1 is observed (8). More recently Wilson et al. (16) determined the three-dimensional structure of influenza HA at a 3 Å resolution. On this basis and considering various types of sequence information a number of residues presumably involved in antigenicity could be identified (17). They were grouped in four domains, and it was proposed that one change in each of the four domains is required to overcome immunity. Considering the comparison Aichi/68-Victoria/75, it may be deduced that in fact 15 of the 22 mutations are part of these four immunogenic domains. It is also of interest to note that as a result of the amino acid changes in HA1 the decoration with carbohydrate groups is not the same for Victoria/75 compared to Aichi/68. This suggests that the structural and functional importance of at least some of these carbohydrate groups is not very stringent.


A/Aichi/2/68
A/NT/60/68/29C
A/Memphis/102/72
A/Victoria/3/75
Observed differences in other natural H3 strains
Observed differences in monoclonal variants
HA1
HA2

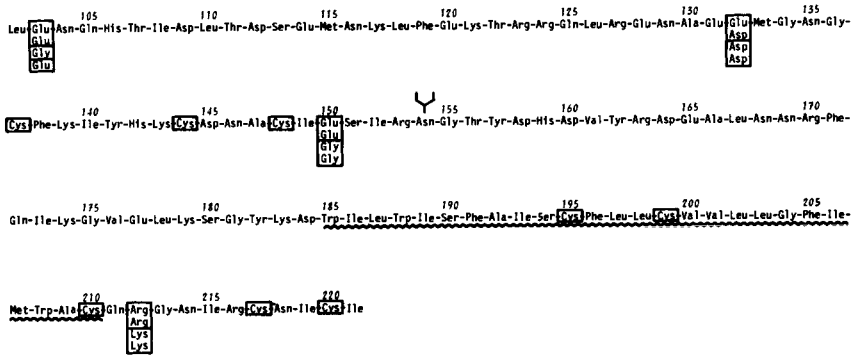


Figure 1. Comparison of influenza A hemagglutinin proteins of the H3 subtype. The amino acid sequence of the hemagglutinin from A/Aichi/2/68 (ref. 8) is shown on top and differences with A/NT/60/68/29C (ref. 10), A/Memphis/102/72 (ref. 9) and A/Victoria/3/75 (ref. 5) are shown in boxes below. These data are all derived from the sequences of cloned DNA. Amino acid changes observed in H3 hemagglutinin by peptide mapping from other field strains (14) and from variants selected by monoclonal antibodies (15) are also indicated. Constant and variable potential glycosylation sites are marked with solid- and broken-lined forks above the relevant asparagine residue. Cysteines, which are constant all over the molecule, are boxed. The wavy line indicates the hydrophobic region of HA2 that is embedded in the lipid bilayer of the virion.

IV. A COMPARISON WITH OTHER HEMAGGLUTININS: SHIFT

The nucleotide sequence of some other influenza hemagglutinin genes has been completely or almost completely established: for Fowl plague virus, which is now classified as H7 (18); the H2 strain A/Japan/305/57 (ref. 11); and an H1 strain, A/WSN/33 (ref. 19). All these quite different sequences can be readily aligned with each other, requiring only three to four insertions/deletions of one or two amino acids. The degree of homology is summarized in Table 1.

It is remarkable that the H3 is longer at the amino-terminal end by 10 amino acids, while the H7 (FPV) has an HA1-HA2 connecting link of five residues rather than one.

TABLE 1. Amino acid sequence homology between different influenza hemagglutinins

	H1[a]	H2[b]	H3	
H2	64 77 69			HA1 HA2 Total
H3	29 50 37	34 50 40	93[c] 98 95	HA1 HA2 Total
H7	28 47 36	36 52 42	37 66 48	HA1 HA2 Total

[a] 32% of H1 has been sequenced.

[b] 83% of H2 has been sequenced.

[c] For comparison the drift between Aichi/68 and Victoria/75 is given.

The HA2 is completely conserved in length, except for an additional residue in the transmembrane region of H1 and H2. It can be seen in Table 1 that the homology between different subtypes is always higher for the HA2 part than for HA1; this again reflects the higher constraints, presumably for functional reasons, on the HA2. Nevertheless, even in the HA1 part there is a high degree of conservation of residues with special structural roles, such as cysteine, proline, glycine, suggesting that the general scaffold remains unchanged.

It is also of interest to note that H1 and H2 are relatively more closely related (Table 1), an indication that perhaps the latter could have arisen from the former by a long drift period. Remarkably, the H3 is even more related to the avian H7 than to the other human hemagglutinins.

V. ON THE ORIGIN OF THE HUMAN H3 SUBTYPE

The H3-type influenza was present in the human population at the turn of the century (20,21) and then disappeared. Much later, in 1968, the Hong Kong flu, which is of the H3N2 type,

swept over the world. What was the origin of this H3 hemagglutinin gene present in this strain? Could it be a reemergence similar to the reemergence of the H1N1 subtype, which reappeared in 1977 almost exactly in the same form as it was present in 1950 (22-24)? The other possibility is that the H3 epidemic started by crossing-in of a hemagglutinin gene from an animal influenza virus into a human strain. Indeed, hemagglutinin which reacted serologically and by hybridization and chemical criteria like H3 could be identified in some animal isolates such as A/Duck/Ukraine/1/63 and A/Equine/Miami/1/63. In order to study this relationship in molecular detail, we have cloned and sequenced the A/Duck/Ukraine/1/63 hemagglutinin gene (25).

The close relationship to the human H3 hemagglutinin could readily be confirmed. The Duck/63 gene had exactly the same length as Aichi/68, and the homology is 90.9% at the nucleotide sequence level and 95.8% at the amino acid level. Again the amino acid changes in the HA1 part are more numerous than in HA2, 4.6% versus 2.3%, reflecting the more stringent structure-function relationship of the latter.

It is of considerable interest to see that Duck/63 is more closely related to Aichi/68 than to Victoria/75. Indeed, the number of amino acid differences is 24 and 40, respectively. This in itself suggests already that these viruses are part of the same genealogical tree. Moreover, among the residues which have been identified as immunologically important 12 are identical between Duck/63 and Aichi/68 while only two are identical between Duck/63 and Victoria/75. It is very likely that the evolution from Duck/63 to Aichi/68 did not occur in humans but rather in an animal, for example duck. Indeed, as described above, by the phenomenon of drift, mutations which change immunologically important positions have a selective advantage in humans and therefore appear more frequently; 15 out of 22 amino acid changes which occurred between 1968 and 1975 are of this class, or a ratio of 0.68. However, only four out of the 15 differences between the Duck/63 HA1 and Aichi/68 change antigenic sites, which means a ratio of only 0.27.

The silent mutations are usually not selected away (except if they have some other function like maintaining a secondary structure or forming other interaction domains, e.g., involved in replication). Assuming then that the accumulation of silent mutations is a simple time-dependent process, we can use the drift between Aichi/68 and Victoria/75 to determine the (silent) mutation rate. On this basis it can be calculated that the common progenitor which gave rise to the Duck/Ukraine/63 virus and to the human Aichi/68 virus was circulating in the animal reservoir around 1949-1953.

The very close homology – even considered in the light of the fast evolution rate of influenza virus – between the animal hemagglutinin from Duck/Ukraine/63 and Aichi/68, which *started*

the Hong Kong flu wave in the human population, provides virtual proof that in this instance a major shift was due to the recombination of an animal hemagglutinin gene into a human influenza virus.

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REFERENCES

1. Fiers, W., Contreras, R., Duerinck, F., Haegeman, G., Iserentant, D., Merregaert, J., Min Jou, W., Molemans, F., Raeymaekers, A., Van den Berghe, A., Volckaert, G. and Ysebaert, M. (1976). *Nature 260*, 500.
2. Devos, R., Van Emmelo, J., Seurinck-Opsomer, C., Gillis, E. and Fiers, W. (1976). *Biochim. Biophys. Acta 447*, 319.
3. Devos, R., Van Emmelo, J., Celen, P., Gillis, E. and Fiers, W. (1977). *Eur. J. Biochem. 79*, 419.
4. Devos, R., Van Emmelo, J., Contreras, R. and Fiers, W. (1979). *J. Mol. Biol. 128*, 595.
5. Min Jou, W., Verhoeyen, M., Devos, R., Saman, E., Fang, R.-X., Huylebroeck, D., Fiers, W., Threlfall, G., Berber, C., Carey, N. and Emtage, S. (1980). *Cell 19*, 683.
6. Gething, M. J., White, J. M. and Waterfield, M. D. (1978). *Proc. Natl. Acad. Sci. U.S.A. 75*, 2737.
7. Schmidt, M. F. G., Bracha, M. and Schlesinger, M. J. (1979). *Proc. Natl. Acad. Sci. U.S.A. 76*, 1687.
8. Verhoeyen, M., Fang, R.-X., Min Jou, W., Devos, R., Huylebroeck, D., Saman, E. and Fiers, W. (1980). *Nature 286*, 771.
9. Sleigh, M. J., Both, G. W., Brownlee, G. G., Bender, V. J. and Moss, B. A., in "Structure and Variation in Influenza Virus" (G. Laver and G. Air, eds.), p. 69. Elsevier North-Holland, New York, (1980).
10. Both, G. W. and Sleigh, M. J. (1980). *Nucl. Acids Res. 8*, 2561.
11. Gething, M. J., Bye, J., Skehel, J. J. and Waterfield, M. (1980). *Nature 287*, 301.
12. Ward, C. W. and Dopheide, T. A., in "Structure and Variation in Influenza Virus" (G. Laver and G. Air, eds.), p. 27. Elsevier North-Holland, New York, (1980).

13. Dopheide, T. A. and Ward, C. W., in "Structure and Variation in Influenza Virus" (G. Laver and G. Air, eds.), p. 21. Elsevier North Holland, New York, (1980).
14. Laver, W. G., Air, G. M., Dopheide, T. A. and Ward, C. W. (1980). *Nature 283,* 454.
15. Laver, W. G., Air, G. M., Webster, R. G., Gerhard, W., Ward, C. W. and Dopheide, T. A., in "Structure and Variation in Influenza Virus" (G. Laver and G. Air, eds.), p. 295. Elsevier North Holland, New York, (1980).
16. Wilson, I. A., Skehel, J. J. and Wiley, D. C. (1981). *Nature 289,* 366.
17. Wiley, D. C., Wilson, I. A. and Skehel, J. J. (1981). *Nature 289,* 373.
18. Porter, A. G., Barber, C., Carey, N. H., Hallewell, R. A., Threlfall, G. and Emtage, J. S. (1979). *Nature 282,* 471.
19. Davis, A. R., Hiti, A. L. and Nayak, D. P. (1980). *Gene 10,* 205.
20. Masurel, N. and Marine, W. M. (1973). *Amer. J. Epidem. 97,* 44.
21. Fedson, D. S., Huber, M. H., Kasel, J. A. and Webster, R. G. (1972). *Proc. Soc. Exptl. Biol. Med. 139,* 825.
22. Kendal, A. P., Noble, G. R., Skehel, J. J. and Dowdle, W. R. (1978). *Virology 89,* 632.
23. Nakajima, K., Desselberger, U. and Palese, P. (1978). *Nature 274,* 334.
24. Scholtissek, C., von Hoyningen, V. and Rott, R. (1978). *Virology 89,* 613.
25. Fang, R.-X., Min Jou, W., Huylebroeck, D., Devos, R. and Fiers, W. (1981). submitted for publication.

CONSERVATION AND VARIATION IN INFLUENZA GENE SEQUENCES[1]

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ABSTRACT

Nucleotide sequences of 20% of the hemagglutinin gene from influenza A viruses representing each of the 12 known subtypes of hemagglutinin show a slow accumulation of nucleotide changes, and some amino acid changes, when viruses of one subtype, isolated over a period of time, are compared. Sequence data from the genes coding for the matrix and non-structural proteins indicate that these genes also drift with time, and the rates of change of amino acid sequences of the gene products are not significantly different from those within HA subtypes. Therefore it seems that antigenic selection does not contribute to the rate of amino acid change.

Between subtypes of hemagglutinin there are very large sequence differences. Certain amino acids, notably the cysteine residues, can be aligned in all strains, and the varying relationships between subtypes which can be seen presumably reflect the evolution of the hemagglutinin from a single gene.

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I. INTRODUCTION

Influenza viruses have two surface antigens, the hemagglutinin (HA) and the neuraminidase. Antibody to the HA neutralises virus infectivity, but because of the variation in HA structure it is not yet possible to produce a vaccine which effectively controls the disease. There are two distinct ways in which the HA can vary: firstly, the year-to-year accumulation of small changes during which the virus becomes progressively insensitive to antibody against the original strain (antigenic drift), and secondly, the sudden appearance of a virus with a hemagglutinin unrelated to the previously circulating strain (antigenic shift).

The new classification of influenza A viruses recently announced by W.H.O. (1) recognises 12 subtypes of HA, based on the absence of any common antigenic determinants detectable in immuno-double-diffusion tests. Within subtypes, viruses have often been isolated from human, animal, and avian hosts, so the HA classification no longer depends on the host from which the virus was isolated, although there can be marked differences in cross-reactivity between viruses of a particular subtype.

Although the HA protein is rather large and difficult to study in detail, the availability of technologies for nucleic acid sequence analysis means that it is now possible to study in detail the sequence variation in influenza virus genes.

The segmented, single-stranded RNA genome can be used to make a cDNA copy of a gene, and this copy inserted into a plasmid for amplification in *E.coli* and nucleotide sequence analysis. Thus the sequences of the fowl plague virus HA gene (H7) (2) and of some human strains, an Asian (H2) type (3) and several Hong Kong (H3) strains (4,5,6) have been determined. From the nucleotide sequences amino acid sequences were predicted using the genetic code.

The sequence of a Hong Kong (H3) HA has also been determined by protein methods (7), and the structure solved at 3Å resolution (8,9).

When the sequences of these three subtypes of HA (H2, H3 and H7) are compared, it is found that the complete lack of antigenic cross-reactivity between them is reflected in a large variation in sequence, at both the nucleotide and amino acid levels.

To study the variation in HA further, we have obtained nucleotide sequence data from the HA genes of each of 35 influenza A viruses, representing each of the 12 known HA subtypes. Rather than clone and completely sequence each HA gene, we have used a 12-nucleotide synthetic primer complementary to the common sequence at the 3' end of all viral RNA segments (10) to prime cDNA synthesis on the HA gene. Using the dideoxy sequencing procedure (11), the length of sequence obtained ranges up to 380 nucleotides, and includes the 5' non-coding region of the mRNA (except for the extra nucleotides derived from cell mRNAs, 12), the signal peptide coding sequence, and the region which codes for up to a third of the HA1 polypeptide. The information for each HA is far from complete, but is a significant proportion and of manageable size for detailed comparison.

A similar study of variation in the neuraminidase and its genes is described by Blok (13). However, it has also been observed that other influenza genes and gene products, which presumably are not under antigenic selection, vary to some extent (14,15), so we have also sequenced cDNA transcribed from the 3' ends of the matrix (RNA Segment 7) and non-structural protein (NS) genes of some of the viruses. The methods and some results have been described previously (16-19).

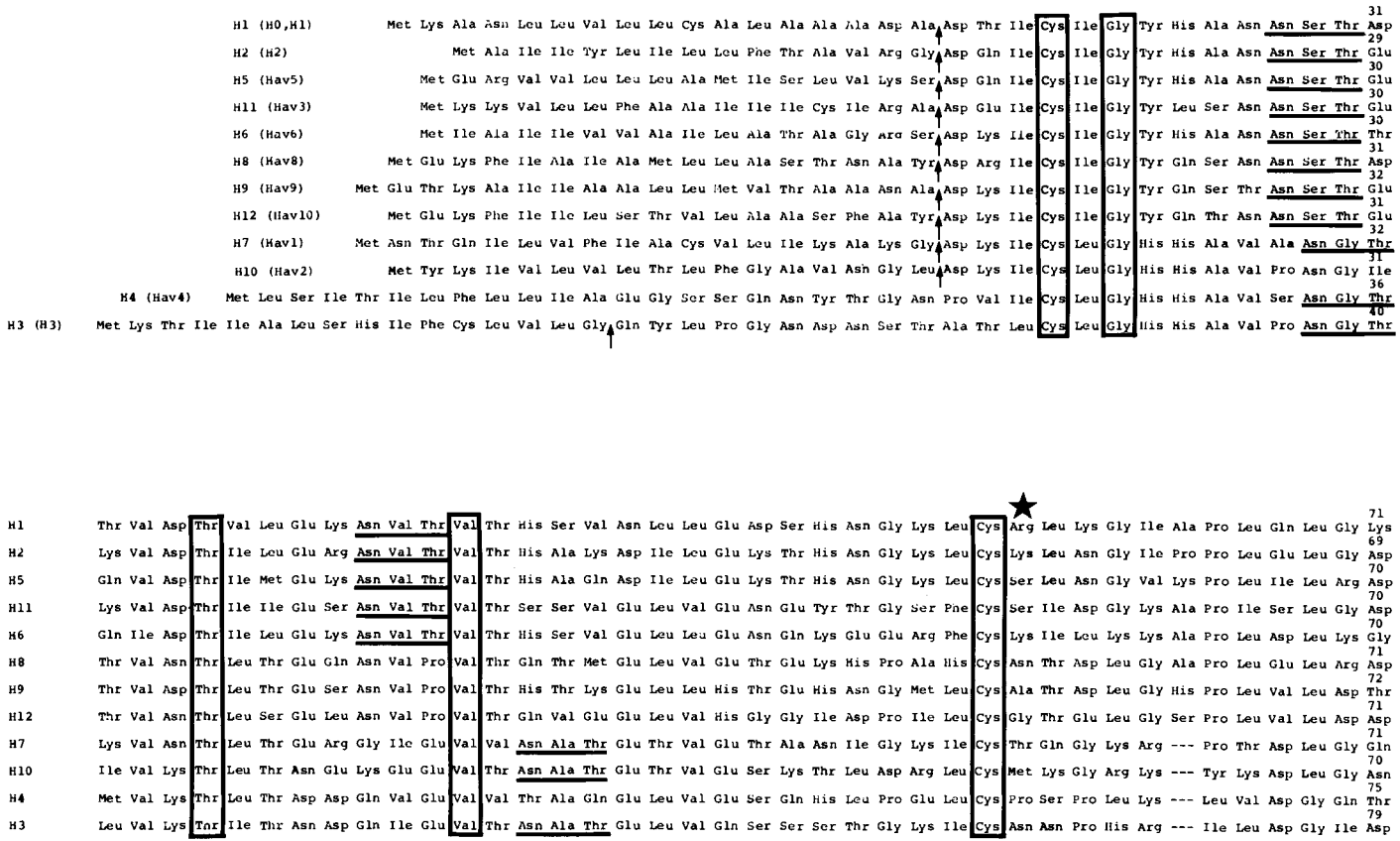
II. RESULTS

A. Sequences From the Hemagglutinin Gene

Figure 1 shows the amino acid sequences predicted from the nucleotide sequences of cDNA transcribed from the 3' end of the HA gene of 12 viruses representing each of the 12 subtypes.

1. The Signal Peptides. In the H2 subtype, the first ATG from the 5' end of the cDNA has been shown to be the start of the signal peptide (16,20,21) and it seems likely that in the other subtypes the first ATG is also the initiating codon. In vivo, the mRNA extends beyond the 3' end of the viral strand RNA, having 10-15 nucleotides including the cap spliced on from non-viral mRNAs (12). The sequences of the extra 10-15 nucleotides vary (22) and it is not known what would happen if they contained an AUG sequence.

The signal peptides contain a central hydrophobic (or non-charged) region which is 11 to 14 amino acids long. When


H1 (H0,H1) Met Lys Ala Asn Leu Leu Val Leu Leu Cys Ala Leu Ala Ala Ala Asp Ala Asp Thr Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Asp 31
H2 (H2) Met Ala Ile Ile Tyr Leu Ile Leu Leu Phe Thr Ala Val Arg Gly Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu 29
H5 (Hav5) Met Glu Arg Val Val Leu Leu Leu Ala Met Ile Ser Leu Val Lys Ser Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu 30
H11 (Hav3) Met Lys Lys Val Leu Leu Phe Ala Ala Ile Ile Ile Cys Ile Arg Ala Asp Glu Ile Cys Ile Gly Tyr Leu Ser Asn Asn Ser Thr Glu 30
H6 (Hav6) Met Ile Ala Ile Ile Val Val Ala Ile Leu Ala Thr Ala Gly Arg Ser Asp Lys Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Thr 30
H8 (Hav8) Met Glu Lys Phe Ile Ala Ile Ala Met Leu Leu Ala Ser Thr Asn Ala Tyr Asp Arg Ile Cys Ile Gly Tyr Gln Ser Asn Asn Ser Thr Asp 31
H9 (Hav9) Met Glu Thr Lys Ala Ile Ile Ala Ala Leu Leu Met Val Thr Ala Ala Asn Ala Asp Lys Ile Cys Ile Gly Tyr Gln Ser Thr Asn Ser Thr Glu 32
H12 (Hav10) Met Glu Lys Phe Ile Ile Leu Ser Thr Val Leu Ala Ala Ser Phe Ala Tyr Asp Lys Ile Cys Ile Gly Tyr Gln Thr Asn Asn Ser Thr Glu 31
H7 (Hav1) Met Asn Thr Gln Ile Leu Val Phe Ile Ala Cys Val Leu Ile Lys Ala Lys Gly Asp Lys Ile Cys Leu Gly His His Ala Val Ala Asn Gly Thr 32
H10 (Hav2) Met Tyr Lys Ile Val Leu Val Leu Thr Leu Phe Gly Ala Val Asn Gly Leu Asp Lys Ile Cys Leu Gly His His Ala Val Pro Asn Gly Ile 31
H4 (Hav4) Met Leu Ser Ile Thr Ile Leu Phe Leu Leu Ile Ala Glu Gly Ser Ser Gln Asn Tyr Thr Gly Asn Pro Val Ile Cys Leu Gly His His Ala Val Ser Asn Gly Thr 36
H3 (H3) Met Lys Thr Ile Ile Ala Leu Ser His Ile Phe Cys Leu Val Leu Gly Gln Tyr Leu Pro Gly Asn Asp Asn Ser Thr Ala Thr Leu Cys Leu Gly His His Ala Val Pro Asn Gly Thr 40

H1 Thr Val Asp Thr Val Leu Glu Lys Asn Val Thr Val Thr His Ser Val Asn Leu Leu Glu Asp Ser His Asn Gly Lys Leu Cys Arg Leu Lys Gly Ile Ala Pro Leu Gln Leu Gly Lys 71
H2 Lys Val Asp Thr Ile Leu Glu Arg Asn Val Thr Val Thr His Ala Lys Asp Ile Leu Glu Lys Thr His Asn Gly Lys Leu Cys Lys Leu Asn Gly Ile Pro Pro Leu Glu Leu Gly Asp 69
H5 Gln Val Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln Asp Ile Leu Glu Lys Thr His Asn Gly Lys Leu Cys Ser Leu Asn Gly Val Lys Pro Leu Ile Leu Arg Asp 70
H11 Lys Val Asp Thr Ile Ile Glu Ser Asn Val Thr Val Thr Ser Ser Val Glu Leu Val Glu Asn Glu Tyr Thr Gly Ser Phe Cys Ser Ile Asp Gly Lys Ala Pro Ile Ser Leu Gly Asp 70
H6 Gln Ile Asp Thr Ile Leu Glu Lys Asn Val Thr Val Thr His Ser Val Glu Leu Leu Glu Asn Gln Lys Glu Glu Arg Phe Cys Lys Ile Leu Lys Lys Ala Pro Leu Asp Leu Lys Gly 70
H8 Thr Val Asn Thr Leu Thr Glu Gln Asn Val Pro Val Thr Gln Thr Met Glu Leu Val Glu Thr Glu Lys His Pro Ala His Cys Asn Thr Asp Leu Gly Ala Pro Leu Glu Leu Arg Asp 71
H9 Thr Val Asp Thr Leu Thr Glu Ser Asn Val Pro Val Thr His Thr Lys Glu Leu Leu His Thr Glu His Asn Gly Met Leu Cys Ala Thr Asp Leu Gly His Pro Leu Val Leu Asp Thr 72
H12 Thr Val Asn Thr Leu Ser Glu Leu Asn Val Pro Val Thr Gln Val Glu Glu Leu Val His Gly Gly Ile Asp Pro Ile Leu Cys Gly Thr Glu Leu Gly Ser Pro Leu Val Leu Asp Asp 71
H7 Lys Val Asn Thr Leu Thr Glu Arg Gly Ile Glu Val Val Asn Ala Thr Glu Thr Val Glu Thr Ala Asn Ile Gly Lys Ile Cys Thr Gln Gly Lys Arg --- Pro Thr Asp Leu Gly Gln 71
H10 Ile Val Lys Thr Leu Thr Asn Glu Lys Glu Glu Val Thr Asn Ala Thr Glu Thr Val Glu Ser Lys Thr Leu Asp Arg Leu Cys Met Lys Gly Arg Lys --- Tyr Lys Asp Leu Gly Asn 70
H4 Met Val Lys Thr Leu Thr Asp Asp Gln Val Glu Val Val Thr Ala Gln Glu Leu Val Glu Ser Gln His Leu Pro Glu Leu Cys Pro Ser Pro Leu Lys --- Leu Val Asp Gly Gln Thr 75
H3 Leu Val Lys Thr Ile Thr Asn Asp Gln Ile Glu Val Thr Asn Ala Thr Glu Leu Val Gln Ser Ser Ser Thr Gly Lys Ile Cys Asn Asn Pro His Arg --- Ile Leu Asp Gly Ile Asp 79

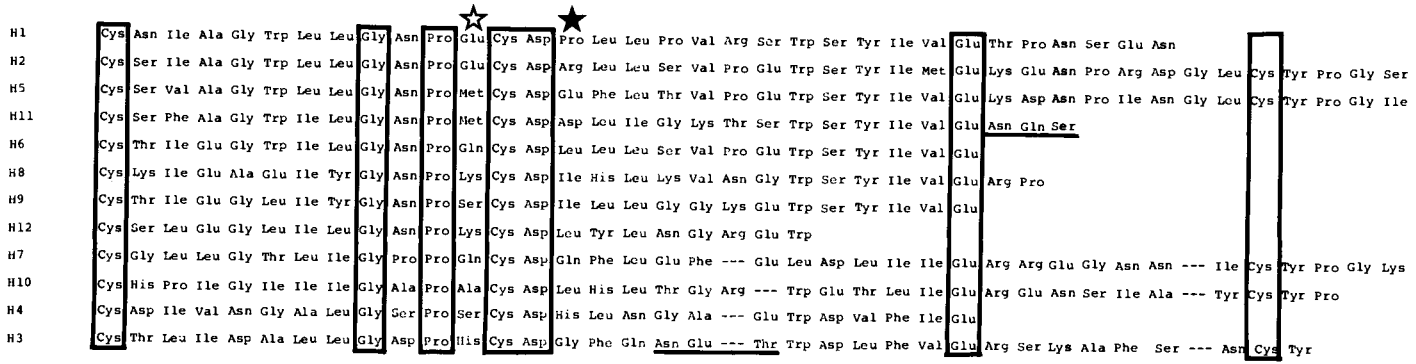


FIGURE 1. Amino acid sequences predicted from sequences of cDNA transcribed from the 3' end of HA genes of viruses representing the 12 HA subtypes. Isolation of HA RNA and sequence analysis of cDNA using the synthetic dodecanucleotide primer (Collaborative Research, kindly given by Dr. C.J. Lai) and the dideoxy method (11) were as previously described (16-19).

The sequences have been aligned at cysteine residues. The N-terminal amino acid of HA1 (actual or presumed) is arrowed, and potential glycosylation sites are underlined. Boxes indicate amino acid residues conserved through all subtypes. ★ marks the two positions where most variation is seen (9 different amino acids), while the position marked ☆ has 8 different amino acids.

The sequences shown are: H1: A/PR/8/34, H2: A/R1/5⁻/57, H3: A/Memphis/1/71, H4: A/duck/Alberta/28/76, H5: A/shearwater/Australia/75, H6: A/shearwater/Australia/72, H7: A/turkey/Oregon/71, H8: A/turkey/Ontario/6118/68, H9: A/turkey/Wisconsin/1/66, H10: A/duck/Manitoba/53, H11: A/duck/Memphis/546/76, H12: A/duck/Alberta/60/76.

these regions are compared, some homology in amino acid sequence can be seen, such as between H5 and H11 and between H8 and H12, but on the whole the only clear similarity is that the signal peptide sequences are very hydrophobic. There is no set pattern of charged amino acids before or after the central hydrophobic portion.

2. The N-terminus of HA1. Viruses of subtypes H1, H2 and H7 have been shown to have Asp at the N-terminus of HA1 (23,24), and since all viruses of all subtypes except H3 and H4 have Asp at the same position (Fig. 1) this may be the N-terminus of the mature HA1 of most subtypes of HA. The H3 N-terminus is

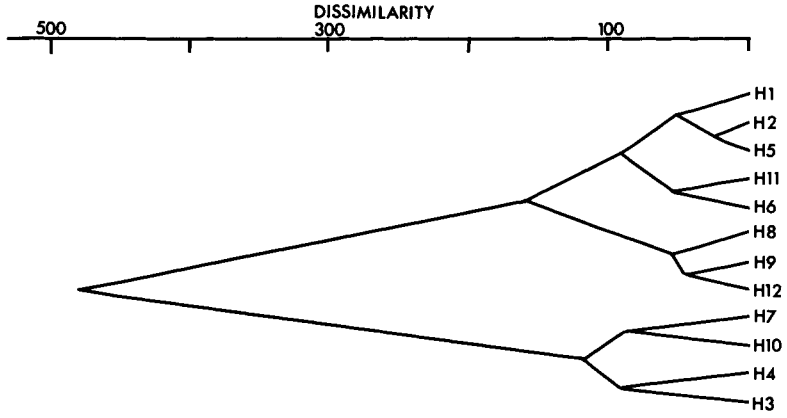


FIGURE 2. Sequence relationships between the 12 HA subtypes. Since the signal peptide sequences are so variable, comparisons have been made from the Asp residue at the N-terminus of H1, H2, H7 (23,24) and of H5, H11, H6, H8, H9, H10, H12 (by homology), or the corresponding amino acid where the N-terminus is longer (H3 (7) and probably H4). The sequences were compared pairwise by diagonal analysis (29) and per cent identity counted, deletions or insertions being counted as mismatches. "Dissimilarity" was then calculated to allow for reversions (30), and the dendrogram calculated using the program of Lance and Williams (31) (Euclidean distance, Burr's strategy).

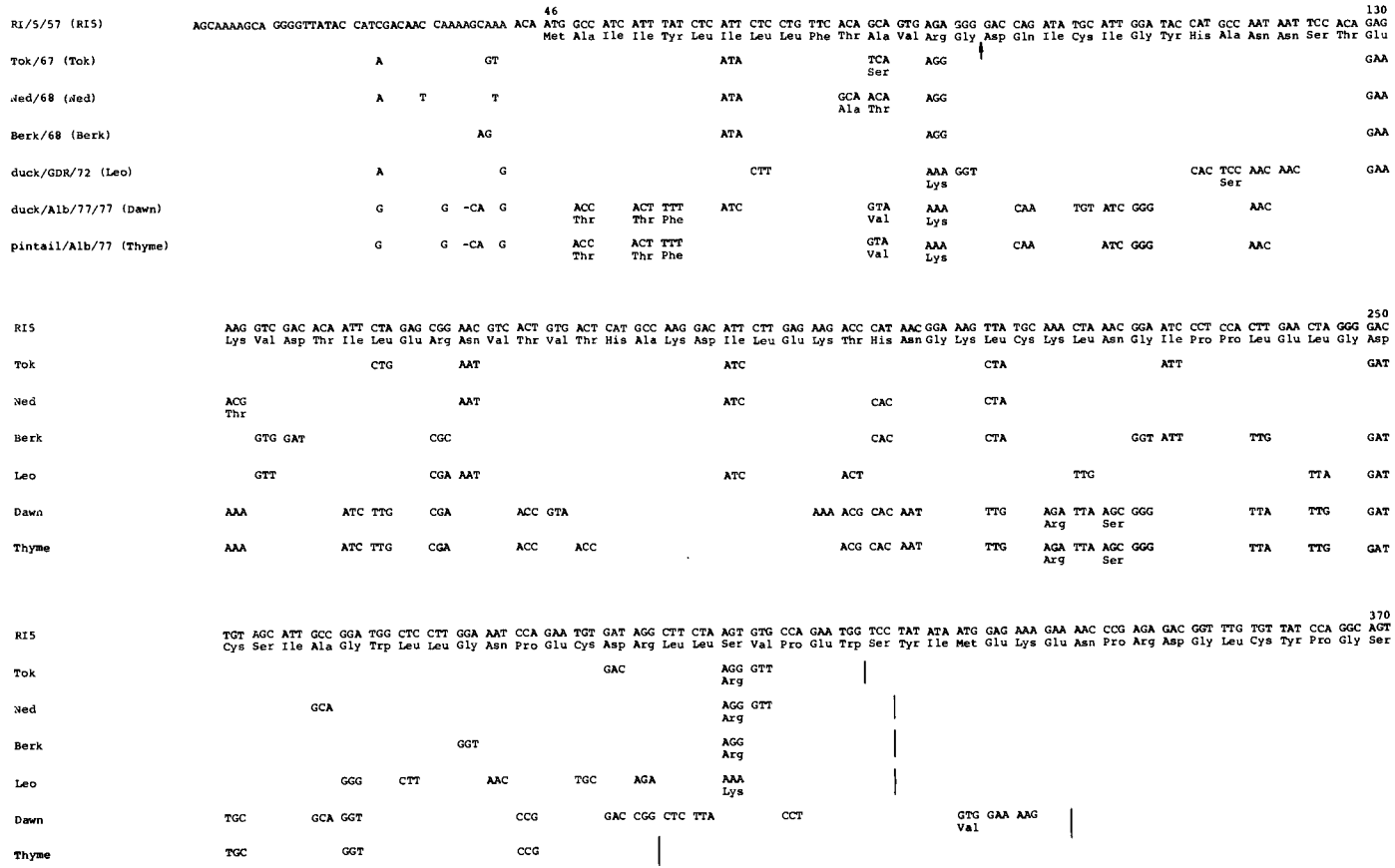
cyclised Gln in Mem/102/72 (7) and this Gln is coded by all other H3 strains sequenced, giving an extra 10 amino acids in these strains. The only subtype where the N-terminus is entirely unknown is H4. Since attempts to directly identify an N-terminal amino acid in this HA1 failed (W.G. Laver, unpublished results), a candidate is the Gln at amino acid 17.

3. Variation in HA1 of Different Subtypes. It is clear from Fig. 1 that although the cysteine residues can be aligned in all subtypes, very few other amino acids are totally conserved. At each of two positions there are 9 and at one position there are 8 different amino acid residues in the 12 sequences, these positions being adjacent to conserved residues. However, some subtypes are more related than others, and an analysis of the relationships is shown as a dendrogram in Fig. 2. The hemagglutinins fall into 2 main families, the "Hong Kong" group (H3, H4, H7 and H10) being very different from the rest. The closest pair are H2 and H5, although no antigenic cross-reactivity can be detected between these.

4. "Drift" Within Subtypes of Hemagglutinin. Fig. 3 shows an analysis of H2 (Asian) strains, isolated at the beginning of the pandemic in 1957 and at the end of the H2 era (1968), and of three "Asian" strains isolated later from birds (1972 and 1977). Although there are many nucleotide changes, only 6 amino acids changed in the N-terminal amino acids of HA1 through this time. Peptides of Ned and Berk have been analysed, and show similar numbers of changes through the rest of the HA1 polypeptide (Laver & Air, unpublished results).

A similar analysis of the cDNA sequences transcribed from the 3' ends of HA genes of the H1 subtype of viruses isolated between 1933 and 1957 showed an accumulation of nucleotide changes but very few amino acid changes in HA1. Two "swine" strains of this subtype, Swine/Wis/15/30 and NJ/11/76 are also highly conserved (25).

It has been recognised for a long time that there is a close relationship between the Hong Kong H3 virus and viruses originally classified as Hav 7 and Heq 2, and these have now all been grouped as H3 (1). From serological and peptide data it has been suggested that animals or birds maintained a "reservoir" of H3 HA genes which reappeared in a human virus in 1968 (26). To investigate the relationships within the H3 subtype, we have obtained sequences from duck/Ukraine/1/63 and another "Hav 7" strain, black duck/Australia/78. Comparisons with human strains are shown in Fig. 4.


46
130
RI/5/57 (RI5)
AGCAAAAGCA GGGGTTATAC CATCGACAAC CAAAAGCAAA ACA ATG GCC ATC ATT TAT CTC ATT CTC CTG TTC ACA GCA GTG AGA GGG GAC CAG ATA TGC ATT GGA TAC CAT GCC AAT AAT TCC ACA GAG
Met Ala Ile Ile Tyr Leu Ile Leu Leu Phe Thr Ala Val Arg Gly Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu
Tok/67 (Tok)
A GT ATA TCA Ser AGG GAA
Ned/68 (Ned)
A T T ATA GCA Ala ACA Thr AGG GAA
Berk/68 (Berk)
AG ATA AGG GAA
duck/GDR/72 (Leo)
A G CTT AAA Lys GGT CAC TCC Ser AAC AAC GAA
duck/Alb/77/77 (Dawn)
G G -CA G ACC Thr ACT TTT Thr Phe ATC GTA Val AAA Lys CAA TGT ATC GGG AAC
pintail/Alb/77 (Thyme)
G G -CA G ACC Thr ACT TTT Thr Phe GTA Val AAA Lys CAA ATC GGG AAC
250
RI5
AAG GTC GAC ACA ATT CTA GAG CGG AAC GTC ACT GTG ACT CAT GCC AAG GAC ATT CTT GAG AAG ACC CAT AAC GGA AAG TTA TGC AAA CTA AAC GGA ATC CCT CCA CTT GAA CTA GGG GAC
Lys Val Asp Thr Ile Leu Glu Arg Asn Val Thr Val Thr His Ala Lys Asp Ile Leu Glu Lys Thr His Asn Gly Lys Leu Cys Lys Leu Asn Gly Ile Pro Pro Leu Glu Leu Gly Asp
Tok
CTG AAT ATC CTA ATT GAT
Ned
ACG Thr AAT ATC CAC CTA GAT
Berk
GTG GAT CGC CAC CTA GGT ATT TTG GAT
Leo
GTT CGA AAT ATC ACT TTG TTA GAT
Dawn
AAA ATC TTG CGA ACC GTA AAA ACG CAC AAT TTG AGA Arg TTA AGC Ser GGG TTA TTG GAT
Thyme
AAA ATC TTG CGA ACC ACC ACG CAC AAT TTG AGA Arg TTA AGC Ser GGG TTA TTG GAT
370
RI5
TGT AGC ATT GCC GGA TGG CTC CTT GGA AAT CCA GAA TGT GAT AGG CTT CTA AGT GTG CCA GAA TGG TCC TAT ATA ATG GAG AAA GAA AAC CCG AGA GAC GGT TTG TGT TAT CCA GGC AGT
Cys Ser Ile Ala Gly Trp Leu Leu Gly Asn Pro Glu Cys Asp Arg Leu Leu Ser Val Pro Glu Trp Ser Tyr Ile Met Glu Lys Glu Asn Pro Arg Asp Gly Leu Cys Tyr Pro Gly Ser
Tok
GAC AGG Arg GTT
Ned
GCA AGG Arg GTT
Berk
GGT AGG Arg
Leo
GGG CTT AAC TGC AGA AAA Lys
Dawn
TGC GCA GGT CCG GAC CGG CTC TTA CCT GTG Val GAA AAG
Thyme
TGC GGT CCG

The amino acid sequence of the N-terminal part of HA1 from duck/Ukraine is very similar to that of the early H3 human strains X31 and Mem/71. However, 4 amino acid differences in 77 and many more nucleotide changes suggest that duck/Ukraine was not a progenitor of the human H3 strains, and perhaps HA genes of other "Hav 7" or "Heq 2" viruses isolated prior to 1968 should be sequenced to see if a closer relationship to the human viruses can be found.

The black duck/78 HA sequence has some amino acids in common with duck/Ukraine (Ser at position 4, Arg at 62), which suggest it is derived from the avian strains rather than a human virus, but there are many nucleotide positions in which it resembles more the human viruses. Even in this example of "drift", where more variation is seen than in the H1 or H2 viruses, there are not enough differences to indicate clearly that black duck/78 is not derived from a human H3 virus.

B. Sequences from the Matrix (M) and Non-Structural Protein (NS) Genes

Much attention has been paid to antigenic variation of the influenza HA and NA (13), and it is possible that antigenic pressure has speeded up the rate of evolution of the HA gene. The data on variation of two other influenza genes, M and NS, which as far as we know are not antigenically significant, suggest that evolution is proceeding at about the same rate without antigenic pressure.

Nucleotide sequences of cDNA transcribed from the 3' ends of RNA segments 7 (matrix gene) and 8 (NS gene) from a number of human influenza A viruses isolated over a period of 43

FIGURE 3. Genetic drift in the HA gene of the H2 subtype of influenza virus. The strains sequenced are identified, together with the shortened or trivial names used in the text. The nucleotide sequence and predicted amino acid sequence are given for cDNA transcribed from the 3' end of RNA segment 4 of R15-. For the other strains, where there is a nucleotide change the whole codon is printed; if this also involves an amino acid change the new amino acid is printed. Blank areas therefore indicate regions where nucleotide and amino acid sequences are identical. Vertical lines indicate the end of data for the strain, and the arrow shows the start of mature HA1.

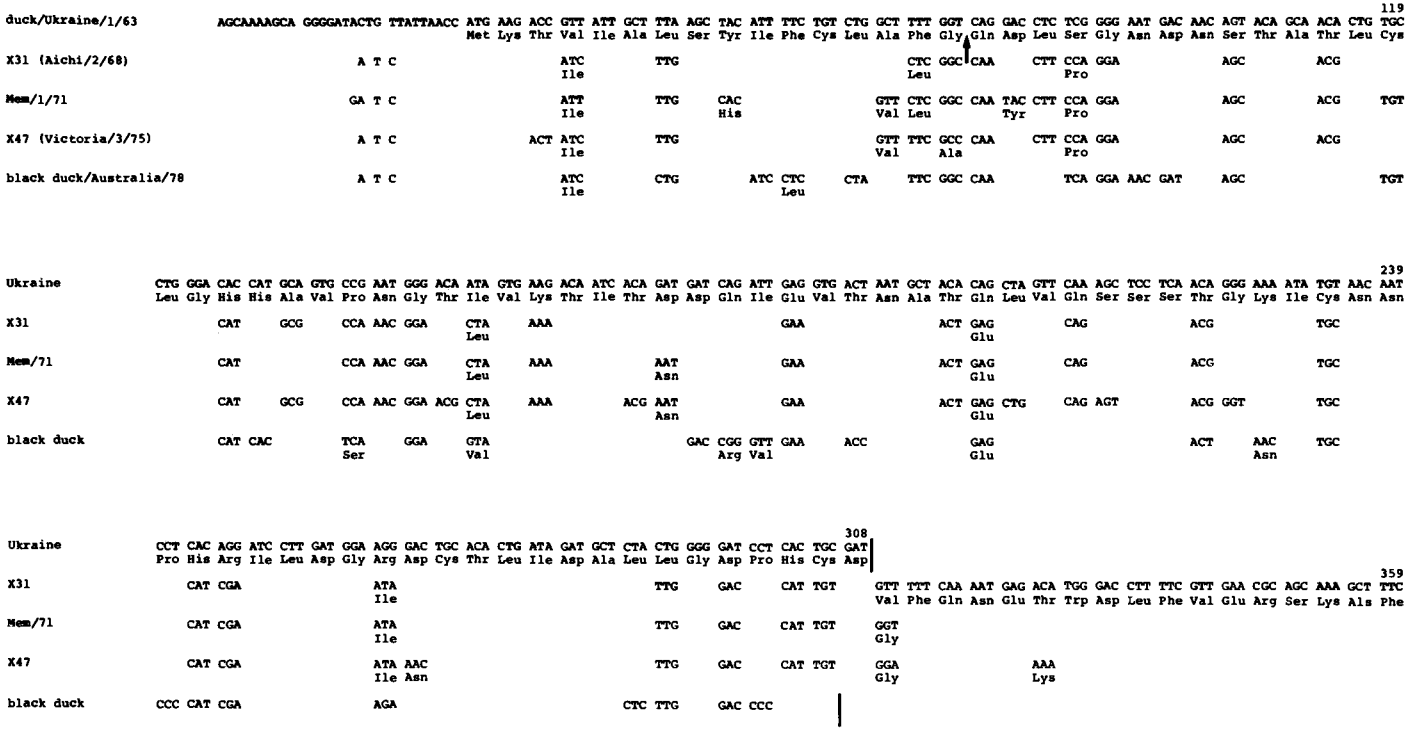


FIGURE 4. Variation in the H3 subtype among human and avian strains. The Aichi/1/68 and Victoria/3/75 sequences are from refs 6 and 5 respectively. Details as in legend to Fig. 3.

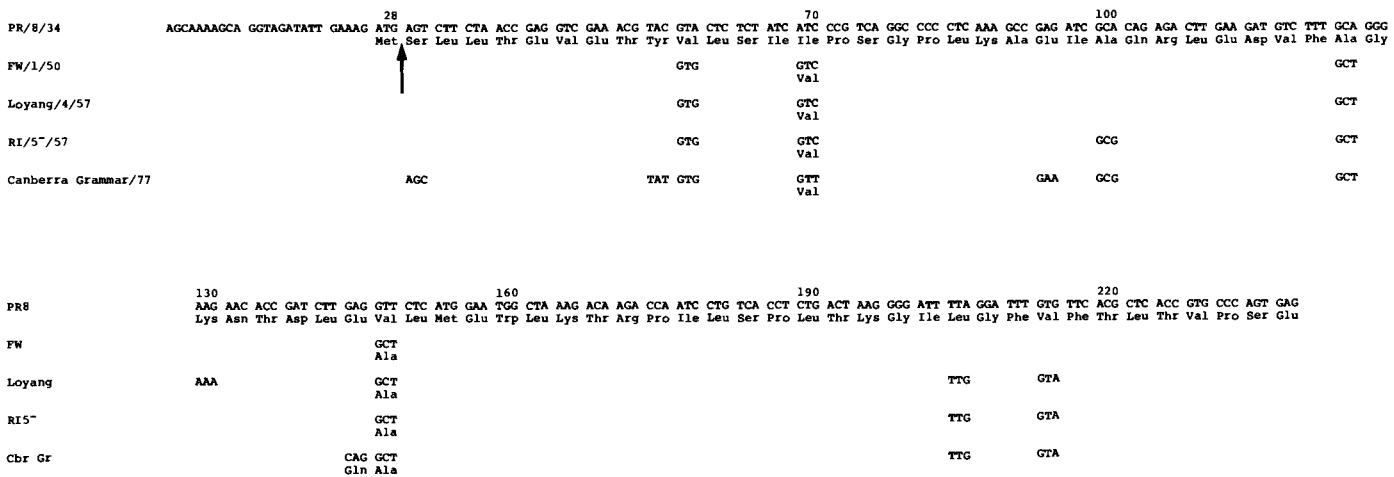


FIGURE 5. Drift in the matrix protein and gene. Details of presentation are as in legend to Fig. 3.

Strain	5′	29 ATG Met	GAT Asp	CCA Pro	AAC Asn	ACT Thr	GTG Val	TCA Ser	AGC Ser	TTT Phe	CAG Gln	GTA Val	GAT Asp	TGC Cys	TTT Phe	CTT Leu	TGG Trp	CAT His	GTC Val	CGC Arg	AAA Lys	CGA Arg	GTT Val	GCA Ala	GAC Asp	CAA Gln	GAA Glu	CTA Leu	110 GGC Gly
PR/8/34	AGCAAAAGCA GGGTGACAAA AACATA																												
RI/5⁻/57	G			CCT											TTC							CAA Gln							GGT
Udorn/72	G			TCC Ser					AGT				GAC		TTC					CGA		CAA Gln		GTA Val					GGT
Canberra Grammar/77	G			TCC Ser					AGT						TTC					AGA		CAA Gln	ATT Ile						GGT
Black duck/78				TCC Ser									GAC										TTT Phe					CTG	GGT
FPV				TCC Ser									GAC										TTT Phe					ATG	GGT

Strain	113 GAT Asp	GCC Ala	CCA Pro	TTC Phe	CTT Leu	GAT Asp	CGG Arg	CTT Leu	CGC Arg	CGA Arg	GAT Asp	CAG Gln	AAA Lys	TCC Ser	CTA Leu	AGA Arg	GGA Gly	AGG Arg	GGC Gly	AGT Ser	ACT Thr	CTC Leu	GGT Gly	CTG Leu	GAC Asp	ATC Ile	AAG Lys	ACA Thr	GCC Ala	ACA Thr	CGT Arg	GCT Ala	GGA Gly	AAG Lys	CAG Gln	ATA Ile	221 GTG Val
PR8																																					
RI5⁻													AAG			AGG		AGA							AAC Asn		GAA Glu			ACC		GTT Val					
Udorn													AAG			AGG		AGA		AGC				CTA	AAC Asn		GAA Glu	GCA Ala		ACC	CAT His	GTT Val					GTA
Cbr Gr							CGA						AAG			AGG		AGA		AGC				CTA			GAA Glu	GCA Ala		ACC	CAT His	GTT Val					
Black duck						GAC		CTC					AAG		CTG			AGA		AGC		CTT					GAA Glu	ACG	GCT	ACT		GTA Val					
FPV						GAC	CGA						AAG		CTG	AGG		AGA		AGC		CTT					GAC Asp		GCT	ACT		GTT Val					

FIGURE 6. Variation in the NS gene. The human viruses show a definite drift with time, while the avian viruses are somewhat different. The Udorn/72 sequence is from ref. 28, that of fowl plague virus from ref. 27.

years and representing H1N1, H2N2 and H3N2 subtypes are shown in Figs 5 and 6.

During the 23 year period between the isolation of PR/8/34 (H1N1) and RI/5⁻/57 (H2N2), substitutions have occurred at 7/230 nucleotides in RNA 7 and 13/220 in RNA 8, and in 20 years (RI/5⁻/57 (H2N2) to CbrGr/77 (H3N2) substitutions have occurred at 5/230 nucleotides in RNA 7 and 12/220 in RNA 8. These give rise to 2/67, 5/64, 1/67 and 5/64 amino acid changes respectively. The number of nucleotide and amino acid changes observed is of the same order of magnitude as occurs over a comparable period of drift in RNA segment 4 and RNA segment 6, which code for the variable antigenic determinants hemagglutinin and neuraminidase.

Hybridization analysis (14) has shown that avian NA genes are somewhat different from those of human strains. The NS sequence of fowl plague virus has been published (27), and we therefore obtained the cDNA sequence from RNA segment 8 of black duck/78. The results are included in Fig. 6, and indicate that whatever the origin of the HA gene, the NS gene of this strain is rather more closely related to avian strains than to human strains.

III. DISCUSSION

A. Genetic Drift in Influenza Genes

The sequencing results presented here show that in influenza genes there is a constant drift, not only in those genes with products under antigenic selection, hemagglutinin (Fig. 3) and neuraminidase (13), but also in those apparently not under antigenic selection, the matrix and NS genes.

Table 1 summarizes the nucleotide and amino acid changes in the M and NS genes, and within subtypes of HA. It is clear that drift proceeds at a measurable rate, although since the virus isolates used probably do not represent a direct lineage it is not possible to calculate this rate with any reliability. If it is attempted, the numbers of nucleotide changes (e.g. per 100 per 20 years) vary by a factor of 5 in the HA genes. However, the proportion of nucleotide changes giving rise to amino acid changes is variable, and although again meaningful calculations cannot be made because the numbers are too small and the origins of the viruses too diverse, there is an impression that the rate of amino acid

change is about the same in all the genes examined.

TABLE I. Nucleotide and Amino Acid Changes During Genetic Drift of Influenza Viruses

Viruses compared	Years difference	Gene	Number of nucleotide differences	Number of amino acid differences
PR8-R15⁻ (Fig 6)	23	NS	13/221	5/65
" (Fig 5)	"	M	7/238	2/71
PR8-Loyang (Fig 5)	23	M	7/238	2/71
" (25)	"	HA	28/314	6/77
Shope-New Jersey(25)	46	HA	30/311	4/76
R15-Ned (Fig 3)	11	HA	16/319	2/77
R15-Thyme (Fig 3)	20	HA	37/305	2/69
R15-CbrGr (Fig 6)	20	NS	12/221	5/65
R15-CbrGr	20	M	5/238	1/71

B. Antigenic Shift

Fig. 1 shows that numbers of amino acid sequence changes between even the closest subtypes are very much larger than any changes seen within subtypes (Figs. 3 and 4). The relationships shown in Fig. 2 are clearly visible in the sequences shown in Fig. 1, but there is still a marked gap between the most dissimilar strains within a subtype and the most similar pair of subtypes (H2 and H5). In most subtypes where several strains have been examined (H1 (25), H2 (Fig. 3) and H3 (6)), there are so few amino acid differences that it is impossible to tell if the subtype is drifting towards any other. In H11 (25), where avian viruses from several bird species from different parts of the world were compared, and the HA sequences show rather more variation, computer analysis does not indicate that the other viruses of the subtype are any more similar to any other subtype than the example used in Fig. 2.

The origins of the 12 HA subtypes (and 9 NA subtypes (13))

are therefore obscure. The relationships seen at the amino acid sequence level are not nearly as obvious when the nucleotide sequences of the HA genes of the various subtypes are examined, and it seems unlikely that the 12 subtypes are the result of the type of drift summarized in Table I. The sequence homologies do indicate a common ancestral gene, and it is possible that the diversification occurred at an early stage of influenza virus evolution. Before the HA developed its present essential role it may not have been under the stringent selection seen now within subtypes, and been able to diversify into 12 stable forms of a functional HA.

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REFERENCES

1. Bull. W.H.O. (1980) *58*, 585-591.
2. Porter, A.G., Barber, C., Carey, N.H., Hallewell, R.A., Threlfall, G. and Emtage, J.S. (1979) *Nature 282*, 471-477.
3. Gething, M.J., Bye, J., Skehel, J. and Waterfield, M. (1980) *Nature 287*, 301-306.
4. Sleigh, M.J., Both, G.W., Brownlee, G.G., Bender, V.J. and Moss, B.A. (1980) *in* "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air eds.), pp. 69-80. Elsevier.
5. Min Jou, W., Verhoeyen, M., Devos, R., Saman, E., Fang, R., Huylebroeck, D., Fiers, W., Threlfall, G., Barber, C., Carey, N. and Emtage, S. (1980) *Cell 19*, 683-696.
6. Verhoeyen, M., Fang, R., Min Jou, W., Devos, R., Huylebroeck, D., Saman, E. and Fiers, W. (1980) *Nature 286*, 771-776.
7. Ward, C.W. and Dopheide, T.A. (1980) *in* "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds.), pp. 27-38. Elsevier.
8. Wilson, I.A., Skehel, J.J., Wiley, D.C. (1981) *Nature 289*, 366-372.
9. Wiley, D.C., Wilson, I.A. and Skehel, J.J. (1981) *Nature 289*, 373-377.
10. Skehel, J.J. and Hay, A.J. (1978) *Nucl. Acids. Res. 5*, 1207-1219.

11. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. 74,* 5463-5467.
12. Plotch, S.J., Bouloy, M. and Krug, R.M. (1979) *Proc. Natl. Acad. Sci. 76,* 1618-1622.
13. Blok, J. (1981) this volume.
14. Scholtissek, C. and von Hoynigen-Huene, V. (1980) *Virology 102,* 13-20.
15. Van Wyck, K.L., Hinshaw, V.S., Bean, W.J. and Webster, R.G. (1980) *J. Virol. 35,* 24-30.
16. Air, G.M. (1979) *Virology 97,* 468-472.
17. Air, G.M. (1980) in "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds.) pp. 135-146. Elsevier.
18. Both, G.W. and Air, G.M. (1979) *Europ.J.Biochem. 96,* 363-372.
19. Air, G.M. and Hackett, J.A. (1980) *Virology 103,* 291-298.
20. Elder, K.T., Bye, J.M., Skehel, J.J., Waterfield, M.D. and Smith, A.E. (1979) *Virology 95,* 343-350.
21. Blobel, G. and Dobberstein, B. (1975) *J. Cell Biol. 67,* 835-851.
22. Lai, C.J., Markoff, L.J., Sveda, M., Dhar, R. and Chanock, R.M. (1980) in "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds.) pp.115-123.
23. Waterfield, M.D., Espelie, K., Elder, K. and Skehel, J.J. (1979) *Brit. Med. Bull. 35,* 57-64.
24. Bucher, D.J., Li. S.S.L., Kehoe, J.M. and Kilbourne. E.D. (1976) *Proc. Nat. Acad. Sci. 73,* 238-242.
25. Air, G.M., Blok, J. and Hall, R.M. in "Replication of Negative Strand Viruses" (D.H.L. Bishop and R.W. Compans, eds.) Elsevier, in press.
26. Laver, W.G. and Webster, R.G. (1973) *Virology 51,* 383-391.
27. Porter, A.G., Smith, J.C. and Emtage, J.S. (1980) *Proc. Natl. Acad. Sci. 77,* 5074-5078.
28. Lamb, R.A. and Lai, C.J. (1980) *Cell 21,* 475-485.
29. Gibbs, A.J. and McIntyre, G.A. (1970) *Europ. J. Biochem. 16,* 1-11.
30. Dayhoff, M.O. (1972) Atlas of Protein Sequence and Structure, Vol. 5.
31. Lance, G.N. and Williams, W.T. (1967) *Aust. Comput. J. 1,* 15-20.

SEQUENCE VARIATION AT THE 3' ENDS OF NEURAMINIDASE GENE SEGMENTS WITHIN AND AMONG THE DIFFERENT NA SUBTYPES

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I. ABSTRACT

Nucleotide sequence studies (150-340 nucleotides) at the 3' end of the influenza neuraminidase gene from 30 strains have shown that there are large differences in the nucleotide and predicted amino acid sequences among the serologically distinct subtypes; and that genetic drift within a subtype is usually the result of point mutations in the RNA gene. Recent studies have revealed that drift also involves deletions and/or insertions, and short regions (about 20 nucleotides) of sequence dissimilarity.

II. INTRODUCTION

The two surface antigens, hemagglutinin (HA) and neuraminidase (NA) of influenza virus undergo antigenic variation which results in serologically different influenza strains, causing pandemics and epidemics. Using immunological techniques, such as double diffusion tests, it has been possible to characterize 12 serologically distinct HA subtypes and 9 NA subtypes (1). Within these subtypes, the surface antigens undergo minor changes which can partially escape antibody neutralization.

In order to study the changes which occur within and among the different NA subtypes, nucleotide sequence studies

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have been carried out on RNA segment 6 (coding for NA). Since the RNA genome of influenza is negative-stranded, the cDNA produced during sequencing by Sanger's dideoxy method (2) is of the same sense as the mRNA. The first ATG (=AUG) may be the initiation site of protein synthesis.

Previous sequence studies on the NA gene (3,4) have shown that the genetic variation which occurs within one subtype correlates well with the antigenic variation (called antigenic drift) as measured by immunological techniques such as neuraminidase inhibition assays. This drift occurs at about the same rate in the HA (5), matrix (major structural protein) (4) and NS (non-structural) genes (6) and it is the result of an accumulation of point mutations in the RNA.

Nucleotide sequence studies of the 12 different HA subtypes have revealed that there are few sequence similarities among the different subtypes (5), which correlates with the lack of antigenic cross-reactivity among these HA subtypes. Two NA subtypes sequenced (3) also showed little homology and recent studies on 8 of the 9 different subtypes (reported here) show a remarkable dissimilarity in NA sequences.

III. METHODS

Influenza viruses (stocks were kindly provided by Dr. W.G. Laver) were grown in embryonated chicken eggs and purified as described in (7). RNA segment 6 was isolated and sequenced using Sanger's dideoxy method (2) with a synthetic dodecamer as previously described (3,6,8,9).

Neuraminidase, the whole molecule or just the pronase-released "heads", was isolated from several virus strains (10, 11). The protein was digested with trypsin and the peptides isolated as in (12), eluted, hydrolyzed and their amino acid compositions determined.

IV. RESULTS

A. Genetic Drift in N1 and N7 - Two Unusual Cases?

Nucleotide sequence studies of the neuraminidase gene have shown that the genetic drift which occurs among the many virus strains within a given subtype is the result of point mutations in the RNA (3,4). A few exceptions to this general

rule have recently been found in the N1 and N7 subtypes.

RNA gene segment 6 (coding for NA) has a variable mobility on polyacrylamide gel electrophoresis, possibly due to a difference in size, among various strains within the N1 subtype. Fig. 1 shows a polyacrylamide gel of the RNA gene segments from 8 N1 strains, isolated from 1930 to 1957, and one N2 strain. The 1930 strain (in channel A) was isolated from pigs while all of the other N1 viruses were human isolates. The nucleotide sequences obtained from the 3' ends of RNA segment 6 from the N1 strains A/swine/Wis/30 (SHOPE), A/PR/8/34 (Mt. Sinai) (PR8), A/Bellamy/42 (BEL), A/FW/1/50 (FW), A/Loyang/4/57 (LOY) and A/USSR/90/77 (USSR) (see Fig. 2) reveal that the difference in mobility of this RNA segment can

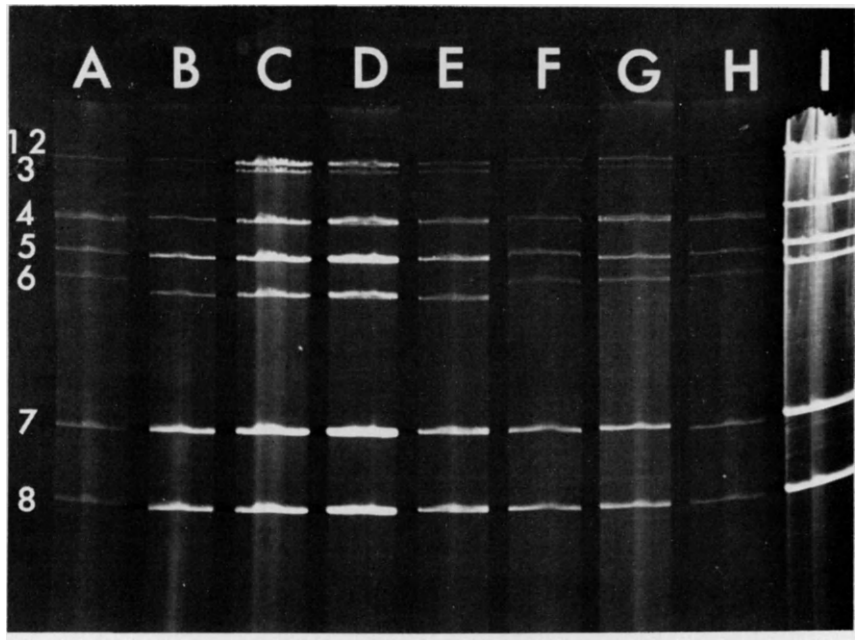

FIGURE 1. A 3% polyacrylamide gel, run for 6 h at 30mA, separating the RNA gene segments of (A) SHOPE (H1N1), (B) A/NWS/33 (H1N1), (C) PR8 (H1N1), (D) A/PR/8/34 (Canberra) (H1N1), (E) A/Melbourne/35 (H1N1), (F) A/Memphis/1/71$_{HA}$-A/Bellamy/42$_{NA}$ (a laboratory recombinant H3N1) (G) FW (H1N1), (H) LOY (H1N1) and (I) A/NWS/33$_{HA}$-A/Tokyo/67$_{NA}$ (a laboratory recombinant H1N2). The migration of segment 6 varies from fast in the 1933-35 N1 strains to slow in the 1930, 1942, 1950 and 1957 N1 strains. The later N1 strains run slightly faster than the N2 to N9 strains.

be explained by change in size since PR8 has a deletion of 45 nucleotides (from 207 to 254 from the 5' end of cDNA) compared with the other N1 strains sequenced.

The neuraminidase gene segment from PR8 has been cloned and sequenced by the "shotgun" method (13), and it has also been partially sequenced from the 3' end (3,14). The nucleotide sequences obtained are identical (except for one nucleotide at position 173) so the deletion is not an artefact of cloning. It remains to be seen if A/NWS/33, A/PR/8/34 (Canberra) and A/Melbourne/35 which appear the same size as PR8 have a deletion in this region.

Amino acid composition analyses of tryptic peptides of BEL neuraminidase have shown that two of the three predicted N-terminal peptides (indicated by the arrows in Fig. 2) can be isolated. One of these peptides spans the region of nucleotides 192 to 254 (or amino acids 57 to 78) so that one can be confident that the reading frame of the nucleotide sequence of BEL is correct in the region where the deletion in PR8 occurs. The other peptide isolated is the N-terminal one, Met-Asn-Pro-Asn-Gln-Lys, which can only be found in the intact NA molecule and not in the pronase-released heads (containing the enzyme activity and antibody binding site) of the same strain. It is therefore likely that the N-terminus is in the membrane-bound tail of the molecule which is cleaved off by pronase.

Fig. 3 shows the nucleotide and predicted amino acid sequence of 3 strains, A/Chick/N/Germany/49 (TOBY), A/duck/Manitoba/53 (MINT), A/equine/Prague/1/56 (EQ) from the N7 subtype. There are many silent base changes among these 3 strains in the first 137 nucleotides from the 5' end of the cDNA. The following region coding for amino acids 39 to 46, is different for 8 consecutive amino acids and somewhere within this region there is a deletion of 3 nucleotides (or 1 amino acid) in MINT and EQ, or an insertion in TOBY. The change in amino acid sequence is not due to a shift in reading frame since the nucleotide sequence is very different among the 3 strains. The sequences following amino acid 47 are similar again including the conservation of two characteristic potential "double" glycosylation sites (Asn-Asn-Thr-Thr). The sequence of EQ, however, has a deletion of 3 nucleotides (205 to 208). Another region lacking homology is located from amino acids 75 to 82 and then the sequences are similar again.

The genetic drift in these N7 strains cannot be compared to that seen in the N1 and N2 subtypes (3) since the N7 strains were isolated from different species, i.e., chicken,

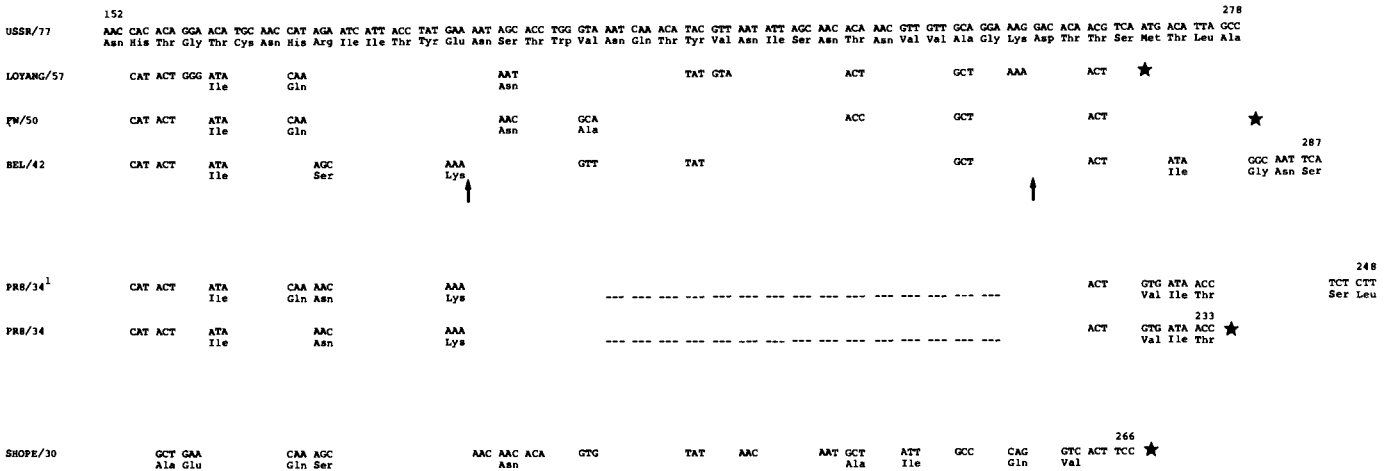

*FIGURE 2. Nucleotide and predicted amino acid sequences of the region 44 to 89 amino acids from the N-terminus of the N1 strains USSR, LOY, FW, BEL, PR8 and SHOPE. Any changes in the nucleotide and predicted amino acid sequence from the top (USSR) sequence have been noted. The * indicates the end of sequence data for that particular strain while --- indicates a deletion of 3 nucleotides. The arrows in the BEL sequence indicate the predicted tryptic peptides. 1 is the PR8 sequenced by cloning the NA gene in M13 (13); while the other PR8 sequence was obtained from the 3' end of the gene. These sequences are identical except for 1 nucleotide, and show a deletion of 45 nucleotides compared with the other N1 strains.*

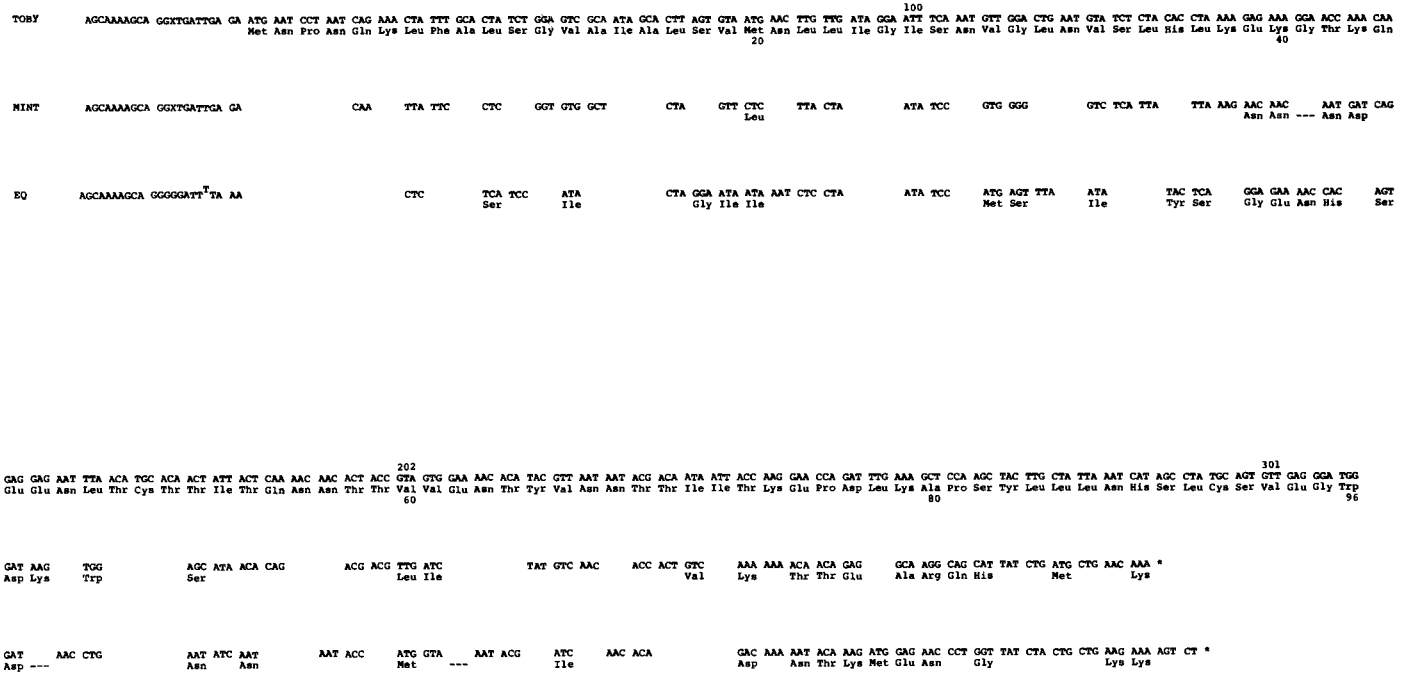

*FIGURE 3. Nucleotide and predicted amino acid sequences of the N7 strains TOBY (H10N7), MINT (H10N7) and EQ (H7N7). Any changes in nucleotide and amino acid sequence from the top (TOBY) sequence have been noted. * indicates the end of sequence data for that particular strain while --- indicates a deletion of 3 nucleotides. The deletion in the region 39 to 46 amino acids from the N-terminus in MINT and EQ is arbitrarily placed. This sequence data represents about 15% of the total gene sequence.*

duck and horse. This may account for the many silent base changes and regions of dissimilarity, but they are distinctly related to each other and can be classified within one serological subtype (N7).

B. Sequence Diversity in the 9 NA Subtypes

The neuraminidase has been classified into 9 serologically distinct subtypes N1 to N9 and various strains from each subtype have been sequenced from the 3' end of the NA gene. Fig. 4 shows the predicted amino acid sequences of 8 strains from 8 of the 9 NA subtypes. Six different strains from the N9 subtype have proved impossible to sequence using a synthetic dodecamer which is complementary to the common sequence at the 3' end of all influenza A RNA gene segments. It may be that the NA gene of this subtype has a base change in this common region thus preventing proper base pairing between the synthetic primer and the RNA.

The first striking feature of the amino acid sequences of the 8 different subtypes is the conservation at the N-terminus. The first 6 amino acids are identical in all of the subtypes while the next 6 are conserved in 5 of the 8 different subtypes. The nucleotide sequence within this conserved region is also very highly conserved where there are only 3 nucleotide changes in the first 15 nucleotides following the ATG. This first ATG ranges from 19-21 to 24-26 nucleotides from the 5' end of the cDNA, depending on the subtype. Following this initial region of conservation, the predicted amino acid sequences change dramatically and this difference is not the result of a small deletion or insertion causing a shift in reading frame because the nucleotide sequences are very different. There is no general conservation of cysteine residues or potential glycosylation sites (Asn-X-Thr, Asn-X-Ser) in the first 80 predicted amino acid residues. This is different from the hemagglutinin (HA) which shows conservation of the cysteine residues and a few other amino acids throughout the 12 HA subtypes (4,5).

V. DISCUSSION

The protein data obtained from the influenza neuraminidase molecule have shown that the N-terminus is in the tail of the molecule which is embedded in the membrane. The presence of a Met in the N-terminal peptide indicates that there is no processing at the N-terminus of the

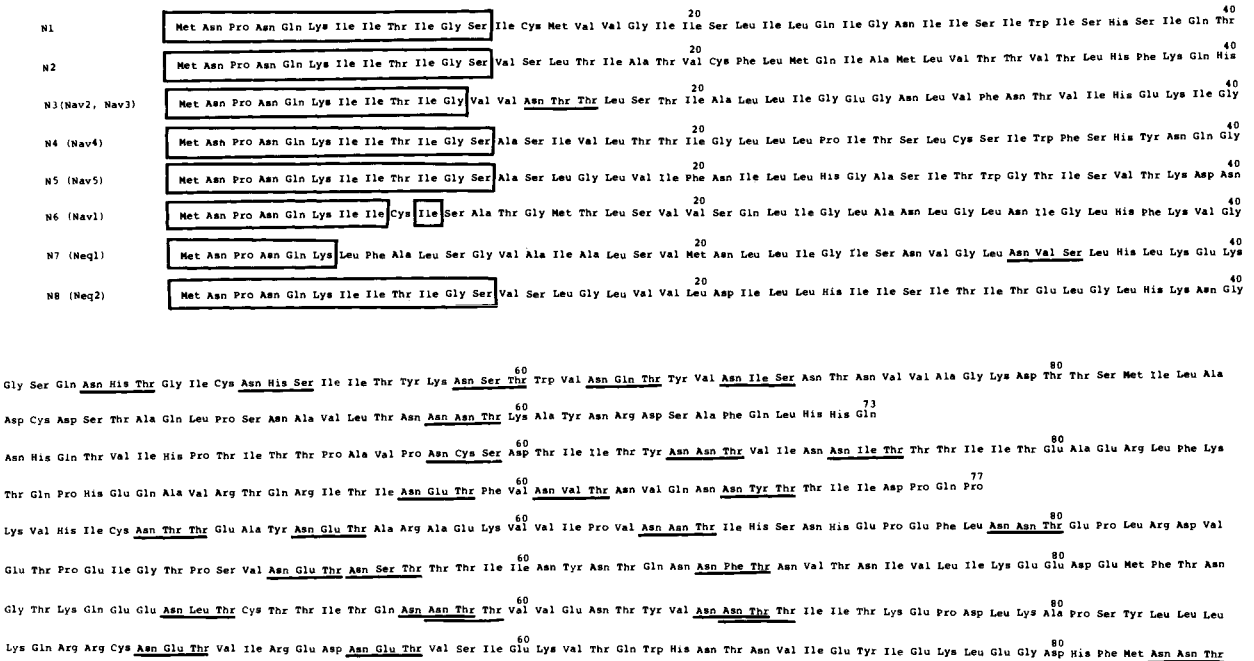

FIGURE 4. Predicted amino acid sequences of one strain from each of the 8 different NA subtypes. The strains sequenced were: A/Bellamy/42 (N1), A/NWS/33$_{HA}$-A/Tokyo/67$_{NA}$ (N2), A/turkey/Oregon/71 (N3), A/turkey/Ontario/6118/68 (N4), A/Shearwater/Australia/72 (N5), A/duck/Alberta/28/76 (N6), A/chick/N/Germany/49 (N7), A/Black Duck/702/78 (N8). The N-terminal region which is conserved in the 8 subtypes has been boxed in. The underlined sequences are the potential glycosylation sites, Asn-X-Ser or Asn-X-Thr.

neuraminidase. In contrast, the influenza hemagglutinin has its C-terminus embedded in the membrane and has a leader sequence at the N-terminus which is cleaved off (15,16).

The region following the first 6 amino acids (which are conserved throughout the 8 NA subtypes) is almost entirely hydrophobic for about 30 amino acids among the different NA subtypes, and then a charged residue, His and/or Lys is present. This hydrophobic segment could be the trans-membrane tail region of the protein and it is then followed by the stalk region which is rich in carbohydrate (17). The sequences of the different subtypes following the His or Lys residues have 1-5 potential glycosylation sites in 40 amino acids (see Fig. 4). The sequences in this region cannot form α-helices (as calculated using the Chou and Fasman method (18)) and there is no conservation of Cys residues either. It can however be envisaged that the stalk, which consists of 4 chains (since the neuraminidase is a tetrameric enzyme), is stabilized by the carbohydrate attached at the glycosylation sites and by the disulfide bonds which may be formed between the Cys residues in each chain. The Cys residues in the tetramer will line up since the chains are identical and therefore the precise location of these residues may not be important as long as they are present.

At the very end of the sequence data obtained so far (about 20% of the total sequence) one can begin to see some conservation of amino acids, in particular around one Cys residue (data not shown). This region is probably in the neuraminidase head which should show some conservation of amino acids (by analogy with the influenza hemagglutinin (5)) in order to maintain enzyme function.

Nucleotide sequence studies (along with amino acid composition data of tryptic peptides) have shown the kind of variation which occurs in the influenza neuraminidase at the molecular level. Genetic drift within a subtype is the result of single base changes in the RNA although the sequence studies have revealed a few exceptions to this involving deletions and insertions. These studies have also shown the totally different sequences which are present in 8 serologically distinct NA subtypes.

VI. ACKNOWLEDGEMENTS

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VII. REFERENCES

1. Memorandum in *Bull. W.H.O.* 58, 585 (1980).
2. Sanger, F., Nicklen, S., and Coulson, A.R., *Proc. Natl. Acad. Sci.* 74, 5463-5467 (1977).
3. Blok, J., and Air, G.M., *Virology*, 107, 50-60 (1980).
4. Air, G.M., Blok, J., and Hall, R.M., *in* "Replication of Negative Strand Viruses" (D.H.L. Bishop and R.W. Compans, eds), Elsevier, in press (1981).
5. Air, G.M., and Hall, R.M., *This Volume*, (1981).
6. Air, G.M., and Hackett, J.A., *Virology*, 103, 291-298 (1980).
7. Laver, W.G., *in* "Fundamental Techniques in Virology" (K. Habel and N.P. Salzman, eds), pp. 82-86. Academic Press, (1969a).
8. Both, G.W., and Air, G.M., *Eur. J. Biochem.* 96, 363-372 (1979).
9. Air, G.M., *Virology*, 97, 468-472 (1979).
10. Laver, W.G., *J. Mol. Biol.* 9, 109-124 (1964).
11. Laver, W.G., *Virology*, 86, 78-87 (1978).
12. Laver, W.G., *in* "Fundamental Techniques in Virology" (K. Habel and N.P. Salzman, eds), pp. 371-378. Academic Press, (1969b).
13. Fields, S., Winter, G., and Brownlee, G.G., *Nature*, in press (1981).
14. Desselberger, U., Zamecnik, P., and Palese, P., *in* "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds), pp. 169-179. Elsevier, (1980).
15. Skehel, J.J., and Waterfield, M.D., *Proc. Natl. Acad. Sci.* 72, 93-97 (1975).
16. Waterfield, M.D., Espelie, K., Elder, K., and Skehel, J.J., *Brit. Med. Bull.* 35, 57-63 (1979).
17. Lazdins, I., Haslam, E.A., and White, D.O., *Virology*, 49, 758-765 (1972).
18. Chou, P.Y., and Fasman, G.D., *Biochemistry*, 13, 222-245 (1974).

INFLUENZA VIRUS A/PR/8/34 GENES: SEQUENCING BY A SHOTGUN APPROACH

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ABSTRACT

We are using a shotgun approach to sequence the genome of influenza virus strain A/PR/8/34, and have now completed the sequences of the genes encoding the haemagglutinin, neuraminidase, matrix protein and non-structural proteins. We prepare double-stranded cDNA corresponding to all the virion RNA segments using synthetic primers for both first and second strand synthesis. The cDNA is then digested with restriction enzymes and the fragments cloned into derivatives of the bacteriophage M13 for sequence analysis by the dideoxynucleotide chain terminator technique.

Highlights of our results so far include: 1)identifying a close sequence relationship between the haemagglutinins of the antigenically unrelated H1 and H2 subtypes; 2)the discovery of a long hydrophobic sequence in the neuraminidase which may orient the molecule with its N-terminus buried in the viral membrane; and 3)finding a second long reading frame in segment 7 which we suggest encodes a novel influenza virus polypeptide.

INTRODUCTION

The biology of influenza A viruses continues to be fascinating, most notably for the antigenic variation occurring in the surface antigens. However, recent studies have revealed other interesting features: viral transcription is primed by short fragments of host mRNAs (1), segment 8 contains two overlapping genes (see ref. 2), and defective interfering RNAs are derived from deletions of the large

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polymerase genes (3). Finally, the mechanism by which the eight RNA segments are selected for assembly into virions remains a mystery. As a basis for a more complete understanding of influenza virus, we are sequencing the entire genome of the strain A/PR/8/34 (H1N1).

The basic strategy we have used is shown in Fig. 1 and described in more detail in refs. 4 and 5. We used a dodecanucleotide complementary to the 3′ end of each virion RNA (vRNA) to prime full-length single-stranded (ss) DNA copies of all the RNA genes. A tridecanucleotide complementary to the 3′ end of the ssDNA was then used to prime second strand synthesis and thus produce full-length double-stranded (ds) DNA copies. We then fragmented the cDNA with a series of restriction enzymes in order to take advantage of a 'shotgun' cloning approach into derivatives of the bacteriophage M13 (6). These M13 clones provide single-stranded templates for rapid sequencing with dideoxynucleotides (7).

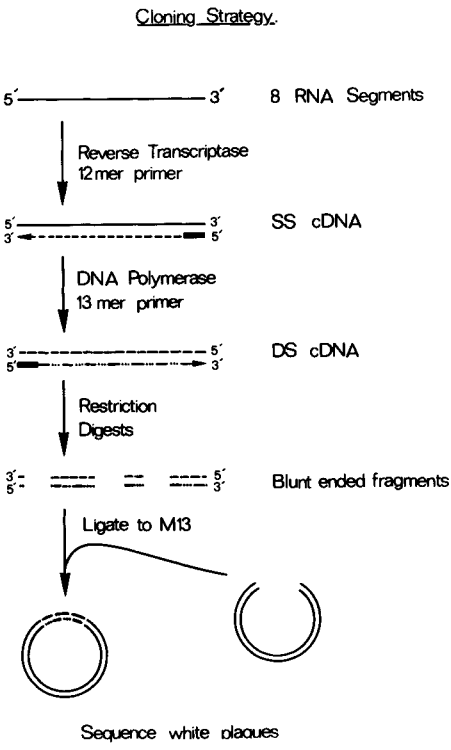

Fig. 1. Shotgun cloning strategy for sequencing the influenza virus genome. The 12-long oligonucleotide d(A-G-C-A-A-A-A-G-C-A-G-G) is used to prime on the vRNA segments in order to produce single-stranded (SS) cDNA. The 13-long oligonucleotide d(A-G-T-A-G-A-A-A-C-A-A-G-G) is used to prime on the single-stranded cDNA to produce double-stranded (DS) cDNA. Restriction digests of the double-stranded material are blunt-end ligated to linearized, blunt-ended M13 replicative form DNA. Recombinant (white) plaques are grown, and the M13 DNA prepared from them in order to obtain influenza virus sequences from throughout the genome.

We have now completed the sequences of the genes encoding the haemagglutinin (see Brownlee et al., this volume), neuraminidase (8), matrix protein (4) and non-structural proteins (5). We present here a summary of the significant features to emerge from the last three of these genes. In addition, we note that a defective RNA (of A/NT/60/68) apparently derived by deletion of segment 3 (B. Moss and G.G. Brownlee, submitted for publication) also contains a region of sequence homologous to the 3′ end of A/PR/8/34 segment 1.

RESULTS AND DISCUSSION

The Neuraminidase - Segment 6

The neuraminidase cleaves N-acetylneuraminic acid (sialic acid) from carbohydrate chains in glycoproteins. Its function may be in removing the carbohydrate residues from the virus envelope itself, thus eliminating receptors for the haemagglutinin and preventing self-aggregation of virion particles (9). Like the haemagglutinin, the neuraminidase can undergo either small changes in antigenicity (antigenic drift) or major changes (antigenic shift) in which the neuraminidases do not cross-react serologically.

Segment 6 encoding the neuraminidase is 1413 nucleotides long, including 20 nucleotides 5′ to the first AUG and 31 nucleotides 3′ to the last coding triplet (8). (The actual structure of the mRNA produced by the virus has the cap and several nucleotides donated by a host mRNA (1) and is an incomplete transcript of the virion RNA (10).) Only one reading frame is open, occupying 97% of the coding capacity of the gene. The predicted protein has 454 amino acids and a molecular weight of 50,087 excuding carbohydrate. Potential carbohydrate attachment sites Asn-X-Ser or Asn-X-Thr (11) occur at 5 positions. These features are indicated in Fig. 2, which shows the positions of the initiation and termination codons, the possible points of carbohydrate attachment, and a region rich in hydrophobic amino acid residues.

The influenza virus neuraminidase is an integral membrane protein and can only be released from the viral envelope by detergent or protease treatment. Previously it was suggested (12) that a hydrophobic region was present at either the N- or C-terminus because the trypsin-released enzyme is smaller than that derived by SDS disruption of the virion. This hydrophobic region would anchor the neuraminidase to the lipid bilayer of the virus. An examination of the A/PR/8/34 neuraminidase protein sequence shows only one major hydrophobic region, located near the N-terminus and extending from amino acid residue 7 to 35. Of these 29

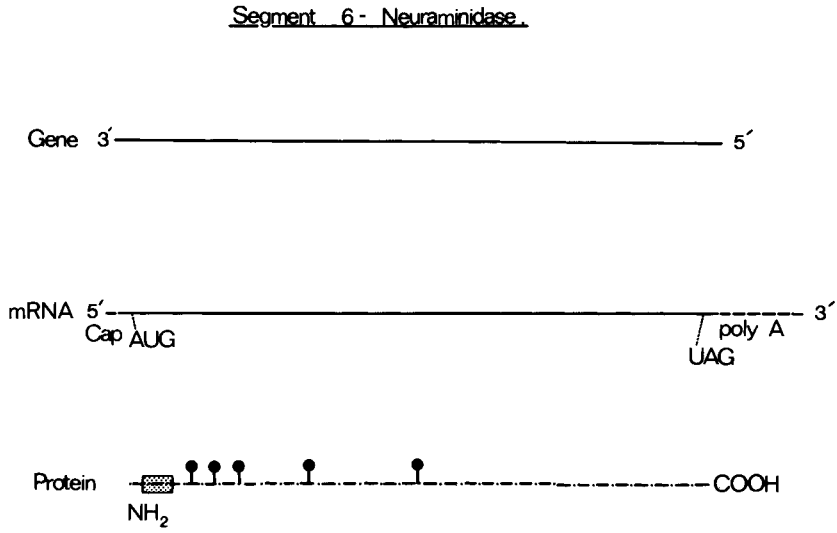

Fig. 2. The neuraminidase gene. The complete sequence of the gene (vRNA) has been determined. The mRNA contains in addition a non-encoded 5′ cap and short region of host-derived nucleotides and a 3′ poly(A) tail. The protein contains a hydrophobic region (shaded) and five potential glycosylation sites (dark circles).

residues, 18 are hydrophobic, 11 are neutral and none is charged. Additional evidence favouring an N-terminal attachment comes from the finding that the small fragment lost on protease isolation of the enzyme from the virion (of FPV) is rich in carbohydrate (13). In the A/PR/8/34 neuraminidase, potential carbohydrate attachment sites near the N-terminus occur at amino acid residues 44 and 58, and at least the first of these sites would be contained within the tryptic peptide presumed to be lost. No attachment sites lie within the C-terminal half of the molecule.

This orientation of the neuraminidase in the membrane would contrast with that of the influenza virus haemagglutinin (14) and also of several other membrane glycoproteins. However, attachment of proteins to membranes via a hydrophobic N-terminus does have precedent in the orientation of intestinal brush border aminopeptidase and isomaltase (15). There is no experimental evidence to indicate whether the N-terminal hydrophobic sequence of the neuraminidase is a 'signal sequence' (16) which is cleaved off, or whether it remains attached to the membrane. However, the almost total conservation in the two human subtypes of the first twelve

amino acid residues (17) suggests that these have some critical role, perhaps in interacting with another protein or neuraminidase subunit. We suggest that the neuraminidase, like isomaltase, has an 'extended signal sequence' (15) which both transfers the protein across the membrane and remains in the bilayer to anchor the protein, with the first six residues protruding into the virion as a hydrophilic tail. This hypothesis could be tested directly by an N-terminal sequence analysis of the intact and trypsin-released enzyme.

The Matrix Protein - Segment 7

Segment 7 encodes the most abundant polypeptide found in virions, the matrix protein, which forms a continuous shell on the inner side of the lipid bilayer. This segment is 1027 nucleotides long, and when the sequence of this gene in its messenger sense is translated, it reveals two long reading frames (4). The first frame encodes the matrix protein (molecular weight 27,861) whose composition predicts a net positive charge of +9.5 at pH 6.5. We speculate that the high positive charge might allow the protein to interact with

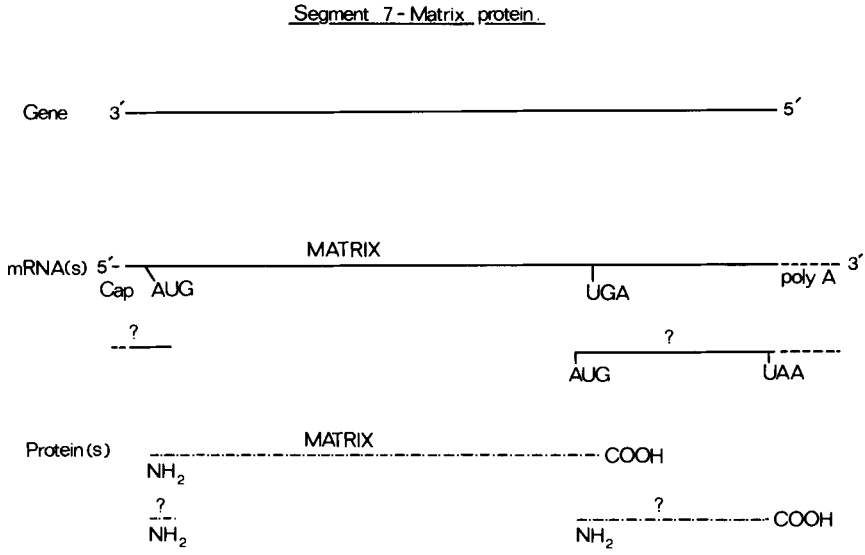

Fig. 3. The matrix protein gene. The sequence of the gene reveals two open reading frames. The first encodes the matrix protein and the second a possible unidentified viral polypeptide. The mechanism of transcription of such a second mRNA remains unknown (see text).

the RNA genome, either in the virion or during packaging.

The second open reading frame in segment 7 overlaps the last 68 nucleotides of the matrix protein frame and continues to a termination codon 23 nucleotides from the end of the segment. It suggests a hitherto unsuspected virally encoded polypeptide. Such overlapping genes have been found in several viruses and have been identified in segment 8 of influenza virus (see below).

A schematic view of segment 7 is shown in Fig. 3. With the presence of the second mRNA and polypeptide in infected cells still unclear, the mechanism of transcription cannot yet be assigned. One possibility is that the second mRNA is produced via RNA processing of the first, as seems likely for segment 8 (2). Another possibility is that the second mRNA is produced by an internal initiation event. The AUG codon beginning the second reading frame is almost immediately preceded by an in-frame termination codon, suggesting that an 11,000 dalton polypeptide is the largest that could be produced by such an internal initiation.

The Non-structural Proteins - Segment 8

The nucleotide sequence of A/PR/8/34 segment 8 (5), like those from A/FPV/Rostock (18) and A/Udorn/72 (2), contains two overlapping reading frames, the first (positions 27 - 719) encoding the NS1 protein and the second (529 - 864) encoding most of the NS2 protein. The two proteins are produced from two different mRNAs, the smaller NS2 mRNA apparently generated from the NS1 mRNA by RNA splicing between positions 56-57 and 528-529 (2). Fig. 4 presents a diagrammatic view of these events, showing the splicing signals which are conserved in all three strains.

A comparison of the amino acid sequence of the NS1 and NS2 proteins from the three strains shows that the NS2 protein is more conserved than the NS1 protein, and that in the overlap of the two reading frames, the NS2 protein is conserved at the expense of the NS1 protein. The third position of each codon in the NS2 frame aligns with the first position of each codon in the NS1 frame, with the result that third position changes in the NS2 frame, which usually leave an amino acid unchanged in character, are likely to provoke radical changes in the NS1 frame.

Two simple models are suggested for the evolution of these overlapping genes. In the first model, the NS1 and NS2 genes are originally colinear on the vRNA, but not overlapping, and with the NS2 mRNA produced by splicing of the large NS1 mRNA. Readthrough of a terminator at the end of NS1 then allows the NS1 protein to encroach into an unused frame of

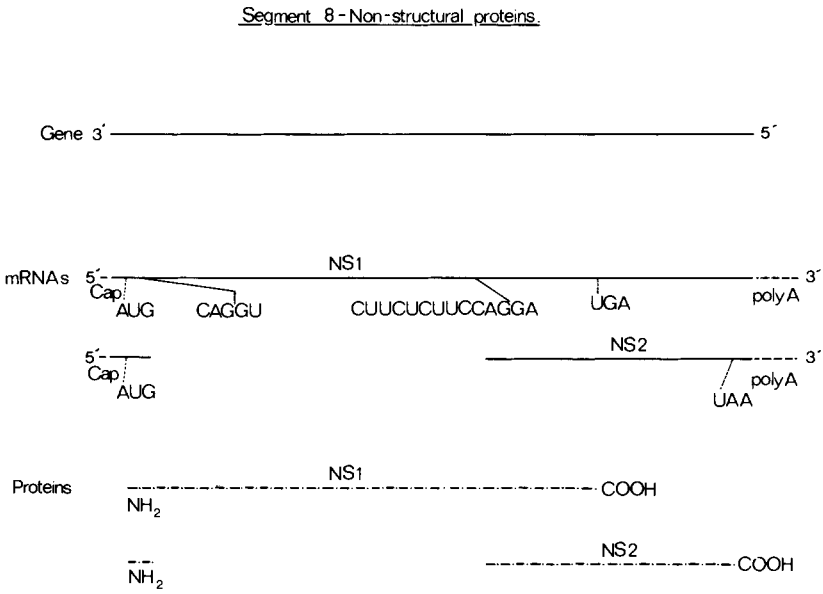

Fig. 4. The non-structural proteins gene. The sequence of segment 8 shows two reading frames, encoding NS1 and NS2. The conserved splicing signals in the NS1 mRNA are indicated. The NS2 mRNA encodes both the common region at the N-terminus of the two proteins, as well as the bulk of the NS2 protein read in a different frame.

the NS2 gene. In the second model, the NS1 protein originally occupies the entire vRNA but as a result of mutations which fortuitously generate a splice site in the NS1 mRNA, a shorter mRNA is produced which encodes the ancestral NS2 protein. In time, the NS2 protein becomes more critical for the function of the virus than the C-terminal portion of the NS1 protein, which is then progressively lost.

The Polymerase Polypeptides - Segments 1, 2 and 3

Sequence analysis of the segments encoding the A/PR/8/34 polymerase polypeptides is not yet complete, but numerous restriction fragments from DNA copies of these genes have been cloned into M13 and sequenced. Among these fragments are those corresponding to the 3' end of the virion RNA of segments 1, 2 and 3. These sequences have been identified by their close homology to the corresponding sequences in FPV RNA as determined by direct RNA sequencing (19).

One fragment from a shotgun digest of cDNA contains

```
 91          101          111
  ATTGGAAGGG TTTTCAGCTG AGTCAAGAAG
  *   * ***      *   *      * **** * *
  AGCGAAAGCA GGTCAATTAT ATTCAATATG
  1           11           21

121          131          141
  GAAAGAATAA AAGAACTACG GAATCTGATG
  ********** ********* *  ***** ***
  GAAAGAATAA AAGAACTAAG AAATCTAATG
 31           41           51

151          161          171
  TCGCAGTCTC GCACTCGCGA GATACTAACA
  ********** **** ***** ****** ***
  TCGCAGTCTC GCACCCGCGA GATACTCACA
 61           71           81

181          191
  ACTGCTTCTT GTCGTTCAGG
  *    *  *
  AAAACCACCG TGGACCATAT
 91          101
```

Fig. 5. Homology between a DI RNA region and a region corresponding to the 3′ end of segment 1 vRNA. The sequence of a region from a cloned DI RNA of A/NT/60/68 is shown above that from a restriction fragment derived from segment 1 RNA. Asterisks indicate nucleotides which match, and the numbering corresponds to the position of each nucleotide in its respective segment. Both sequences are written in the mRNA sense.

about 300 nucleotides which match and extend the limited sequence determined at the 3′ end of vRNA segment 1 (19). In addition, a portion of this sequence matches part of the sequence of a small, defective interfering (DI) RNA from A/NT/60/68 whose 5′ and 3′ termini are those of segment 3 (B. Moss and G.G. Brownlee, submitted for publication); this match is shown in Fig. 5. This unexpected homology suggests as one possibility that segment 3 contains one region which is 90% homologous in nucleotide sequence to segment 1 (strain differences may account for some of the mismatches). Another more intriguing possibility is that the DI RNAs might arise by not only deletion but inter-segment splicing. These possibilities will be clarified when the complete sequences of the polymerase genes become available.

Sequence Heterogeneity

The M13 shotgun approach has allowed us to look at heterogeneity within the RNA population, since an average sequence is obtained which reflects a number of individual RNA segments. Although the surface proteins undergo changes in antigenicity, sequence analysis of these genes has shown very little variability within this RNA population (8).

An independent sequence of the gene encoding the A/PR/8/34 matrix protein (20) shows 6 differences from our sequence. Similarly, another sequence of segment 8 of A/PR/8/34 (21) shows 10 differences from the one we determined. These figures presumably reflect sequence drift in the different stocks of virus used in the sequence determination.

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REFERENCES

1. Krug, R.M. et al. (1979) Cell 18, 329.
2. Lamb, R.A. and Lai, C.-J. (1980) Cell 21, 475.
3. Nayak, D.P. (1980) Ann. Rev. Microbiol. 34, 619.
4. Winter, G. and Fields, S. (1980) Nucleic Acids Res. 8, 1965.
5. Winter, G. et al. (1981) Nucleic Acids Res. 9, 237.
6. Sanger, F. et al. (1980) J. Mol. Biol. 143, 161.
7. Sanger, F. et al. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463.
8. Fields, S. et al. (1981) Nature, in press.
9. Palese, P. et al. (1974) Virology 61, 397.
10. Skehel, J.J. and Hay, A.J. (1978) Nucleic Acids Res. 5, 1207.
11. Neuberger, A. et al. (1972) In The Glycoproteins: Their Composition, Structure and Function, A. Gottschalk, ed. (Amersterdam: Elsevier), p. 450.
12. Lazdins, I. et al. (1972) Virology 49, 758.
13. Rott, R. et al. (1972) Z. Naturforsch. B. 27, 227.
14. Skehel, J.J. and Waterfield, M.D. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 93.
15. Frank, G. et al. (1978) FEBS Lett. 96, 183.
16. Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 835.
17. Blok, J. and Air, G.M. (1980) Virology 107, 50.

18. Porter, A.G. et al. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5074.
19. Robertson, J.S. (1979) Nucleic Acids Res. 6, 3745.
20. Allen, H. et al. (1980) Virology 107, 548.
21. Baez, M. et al. (1980) Nucleic Acids Res. 8, 5845.

THE HAEMAGGLUTININ GENE OF INFLUENZA A/PR/8/34

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ABSTRACT

The complete nucleotide sequence of the haemagglutinin gene of influenza strain A/PR/8/34 (H1 subtype) was determined by "shotgun" cloning of restriction fragments derived from gene 4 into the bacteriophage M13 and sequencing with dideoxy chain terminators. From the deduced amino acid sequence of the haemagglutinin we define an antigenic site at amino acid residue 160. A comparison of the amino acid sequence of the haemagglutinins of different influenza A strains shows that the H1 and H2 subtypes are more closely related to one another than either subtype is to the H3 subtype or H7 subtype.

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INTRODUCTION

Influenza remains a serious cause of disease in Man and of death in the aged. The prevention of infection by vaccination, so effective in other viral diseases such as poliomyelitis and measles, is only transiently effective in providing immunity in influenza (1). This is because the surface haemagglutinin molecule can undergo changes in antigenicity which allow virus to escape neutralization by immunizing antibody.

Of the 3 subtypes H1, H2 and H3 (for new nomenclature see ref 2) known to cause influenza A infection in Man, the H1 subtype is of particular interest as it, or its variants, have been the dominant human subtype this century. After isolation in 1933, it persisted until 1957: then following a dormant period of 20 years, it re-emerged as the "Russian flu" in 1977 and is now cocirculating with variants of the H3 (Hong Kong) subtype. Moreover, it probably caused the serious pandemic of 1918 (3). Previous detailed structural studies of the haemagglutinin have been restricted to the H7 subtype (4) (fowl plague) and to the later H2 and H3 human subtypes (5-10). Here we determined the sequence of the haemagglutinin of an early H1 subtype (strain A/PR/8/34, formerly designated HO), using the rapid M13 cloning and dideoxy sequencing methods developed by Sanger (11,12). We compare this with the sequences of other haemagglutinins from other subtypes and also define the position in the primary structure of an antigenic site in the H1 subtype. From this analysis it emerged that the H1 and H2 subtypes show a closer homology to one another than to other subtypes, suggesting they have diverged from one another recently.

METHODS

Influenza is a negative-stranded virus with 8 RNA genes coding for 10 or more proteins, of which band 4 codes for the haemagglutinin gene. The main strategy for sequencing this gene was similar to that used in a parallel study of the other influenza genes (13-15 and Fields & Winter, this volume). We prepared restriction fragments derived from double stranded DNA, itself prepared by <u>in vitro</u> copying of the RNA gene 4 with reverse transcriptase. These restriction fragments were cloned into the bacteriophage M13 vector and sequenced by the

Sanger dideoxy method. By sequencing clones which were isolated at random and derived from a number of different restriction enzyme digests, we hoped to overlap these clones and deduce a unique sequence for the haemagglutinin gene.

Initially as part of our aim for sequencing all the genes of influenza (13-15) we used a mixture of all 8 virion RNA genes of strain A/PR/8/34 (kindly supplied by Dr B Mahy, Dept. Virology, Univ. Cambridge) as starting material for preparing clones. Double-stranded DNA synthesised in vitro was digested with the restriction endonucleases Alu I, Sau 3A and Taq I and cloned in M13mp2, M13mp2(Bam) or M13mp7 (16-18). After dideoxy sequencing using a universal M13 primer, influenza gene sequences were collected and collated using the data handling programs of Staden (19) on a PDP/11 computer. Amongst this data we tentatively assigned a number of clones to the haemagglutinin gene either by virtue of an overlap extending the 5' terminal sequence previously determined by Air (20) or by virtue of significant homology of the predicted amino acid sequence derived from these cloned sequences to that of the H2 subtype (5). With this "random shotgun" (13) approach about 65% of the haemagglutinin gene was cloned in non-overlapping sections, which (by chance) corresponded mainly to the HA1 subunit portion of the molecule.

Sequence information obtained by the shotgun approach was extended in a second approach by preparing clones derived from the purified haemagglutinin gene. We found it more convenient to purify the full length band 4 complementary DNA, derived by reverse transcription of the mixture of all 8 virion components (21) rather than purify the specific band 4 RNA. The use of the 12-long primer d(A-G-C-A-A-A-A-G-C-A-G-G) (22) gave markedly improved yields of full length cDNA than previously obtained by polyadenylating the RNA and priming with oligo $d(T_{12-18})$ (13). Band 4 cDNA was converted to double-stranded DNA in vitro using the 13-long deoxy-oligonucleotide primer d(A-G-T-A-G-A-A-A-C-A-A-G-G) (15) complementary to the 3'-terminus of the single stranded DNA. Restriction digests of this material were cloned as before (14). Using this "band 4 shotgun" method and in conjunction with the results of the random shotgun method, we sequenced 32 unique clones which covered all residues in the finally deduced structure (Fig 1) except residues 1398-1412 where we were unable to isolate a suitable overlapping clone. As clones were present in both orientations in M13, 76% of the sequence was cross-checked on both strands of the DNA. Of the remainder, only 10% was not sequenced from independent clones. The sequence of residues 1398-1412 was determined using an indirect RNA sequence method (35). A restriction fragment corresponding to residues 1222-1273 was isolated from an R_F

preparation of a suitable recombinant haemagglutinin clone. This fragment was used as a primer for dideoxy sequencing using reverse transcriptase and total virion RNA as template (13).

Finally there remained the uncertainty that the 13-long oligonucleotide primer used for preparing double stranded DNA might not be exactly complementary to the template strand. We therefore checked the sequence at the 5' end of the band 4 virion strand by direct RNA sequencing using the method of partial enzymatic cleavage (36). We thus established the correctness of the residues corresponding to the 13-long primer. The sequence corresponding to the 12-long primer had been independently checked in cloning of poly(A) - containing restriction fragments in our first approach.

RESULTS AND DISCUSSION

Structure of the Haemagglutinin Gene

Fig 1 shows the nucleotide sequence deduced for the haemagglutinin gene written in its mRNA sense. 99% of this sequence was derived by the "shotgun" restriction fragment cloning; the remainder required the indirect RNA sequencing methodology (see Methods). The gene is 1778 nucleotides long coding for a protein of 566 amino acids. There is only a short non-coding region at either end of the molecule containing within them the well known 'conserved' 12 and 13-long partially complementary sequences (23). We find no evidence of alternative "reading frames" coding for another protein, either from the mRNA strand or from the complementary virion strand. Consistent with its lack of any introns, there are no obvious splicing recognition sequences as occurs for example in the influenza gene 8 RNA sequence (24). The entire mRNA sequence is A rich (A=35%, U=24%, G=23%, C=19%), and this is reflected in the frequency of codon usage (data not shown) where A is generally, but not always preferred in the third position. As has been noted before (4), C-G is rarer than expected and for example the codons C-G-X (where X is A, G, C or U) are not used for arginine whereas A-G-G and A-G-A are used 10 and 11 times respectively.

The sequence of residues 1-249 of A/PR/8/34 determined by Air (20) differs from that presented here at nucleotides 69, 140 and 161. While the base change at position 69 (C→U) changes the amino acid residue from alanine → valine, the changes at the other two positions (G → A) are "silent".

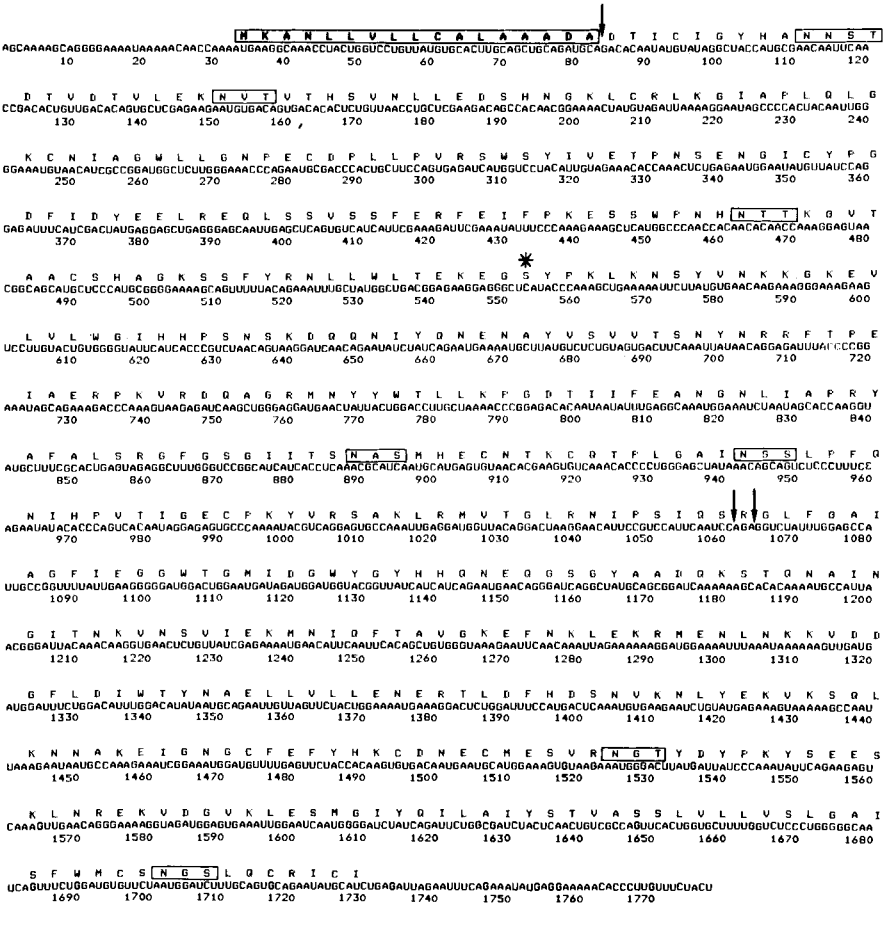

FIGURE 1. The nucleotide and amino-acid sequence of the haemagglutinin gene from A/PR/8/34 (Cambridge strain) written in the mRNA sense. The cleavage points at the N-terminus of the HA1 and the HA1/HA2 junction are marked with an arrow. Potential glycosylation sites are boxed, while the signal peptide is boxed and shaded. The asterisk marks the Ser → Leu change in an antigenic variant (see text). From ref 37 with permission.

These differences may reflect real variations in substrains of A/PR/8/34 used in different laboratories. Our own sequence, at least at position 69, is derived from two independent clones, so is unlikely to derive from a minor variant of our A/PR/8/34 population.

Structure of the Haemagglutinin

The general organization of the haemagglutinin of A/PR/8/34 is very similar to that of haemagglutinin molecules previously studied in other subtypes (4-9,25). We can identify a 17-long hydrophobic signal peptide, an HA1 subunit (326 residues long) and an HA2 subunit (222 residues long). These, as in the other human subtypes, are separated by a single arginine residue (327) which is presumably recognized during processing by a trypsin-like enzyme. The HA2 subunit contains the characteristic conserved 14-long hydrophobic N-terminal sequence as well as a hydrophobic tail, believed to anchor the molecule to the phospholipid bilayer of the cell (26).

Our studies provided no direct information on the secondary structure of the haemagglutinin, but we can deduce from the conservation of cysteine residues (Fig 2) that the pattern of disulphide bonds is probably similar to that of the H2 and H3 subtypes where most cysteines are involved in S - S bridges within a subunit except for C(14) and C(466) (see Fig 2) which interconnect the separate subunits (27). There is no information on the three conserved cysteine residues close to the C-terminus of the HA2 subunit. The haemagglutinin is a glycoprotein and we observe 7 potential N-X-S or N-X-T sequences (Fig 1) where carbohydrate could be attached (28). Direct studies on the location of the carbohydrate in A/PR/8/34 are lacking but studies of related strains (A/WSN/33) (29) and other subtypes (A/Japan/57) (5) suggest that most sites are glycosylated. Presumably the most N-terminal glycosylation site in the HA1 subunit (N-N-S-T - a sequence also conserved in the H2 subtype) is glycosylated at one but not both of the asparagine residues (5).

Antigenic Variation in the H1 Subtype

Monoclonal antibodies have been previously used to select A/PR/8/34 variants which escape antibody neutralization because of their changed antigenicity (30). A frequently observed variant contained a leucine residue replacing a serine residue in the parental tryptic peptide (S,E,P,G,Y)K, although this peptide could not be further localized within

the sequence. From the amino acid sequence predicted here (Figs 1 and 2), we identify this variant as serine → leucine (160) caused by a C → U transition at residue 553. Residue 160 has not previously been implicated as an antigenic site in the better studied H3 subtype, despite the fact that the homologous residue threonine (160) appears to be on the surface of the haemagglutinin in the recently available three-dimensional model of the H3 subtype (31). Perhaps the presence of the nearby carbohydrate side chain at residue 165 masks this region in the H3 subtypes. Further studies of other laboratory variants as well as field strains with altered antigenicity is required, however, to define the antigenic region(s) of the H1 subtype in more detail.

Homology between different subtypes

Figure 2 shows a comparison of the amino acid sequence of the A/PR/8/34 haemagglutinin with representative strains of 3 other subtypes. Only occasional gaps were inserted in one or other of the sequences to maintain alignment. This confirms the basic homology of all haemagglutinin subtypes. The fact that cysteine and many proline residues are conserved argues for the importance of these residues in the folding of the protein and predicts a common overall shape. The presence of common features is important also as it eliminates these residues from participation in antigenic variation. Some at least of the common residues must be important in the early stages of infection by influenza involving host cell receptor binding of the haemagglutinin to the cell membrane.

Table 1 presents a quantitative measure of the extent of amino acid similarity of the different subtypes, and shows that the H1 and H2 subtypes are more similar to one another in both their HA1 and HA2 subunits than either is to the H3 or H7 subtypes. The 58% amino acid conservation in the HA1 subunit of the H1 and H2 subtypes is far greater than the typical 33-36% similarity of the other possible comparisons.

Evolution of Subtypes

The demonstrable homology of all haemagglutinin subtypes shows that they must have evolved from a common ancestral gene by a process of point mutation and the occasional deletion and addition. We know from a study of the extent of antigenic drift in the human H3 subtypes that change can occur quickly at a rate which is in the order of 1% amino acid change per year for the HA1 subunit (a value

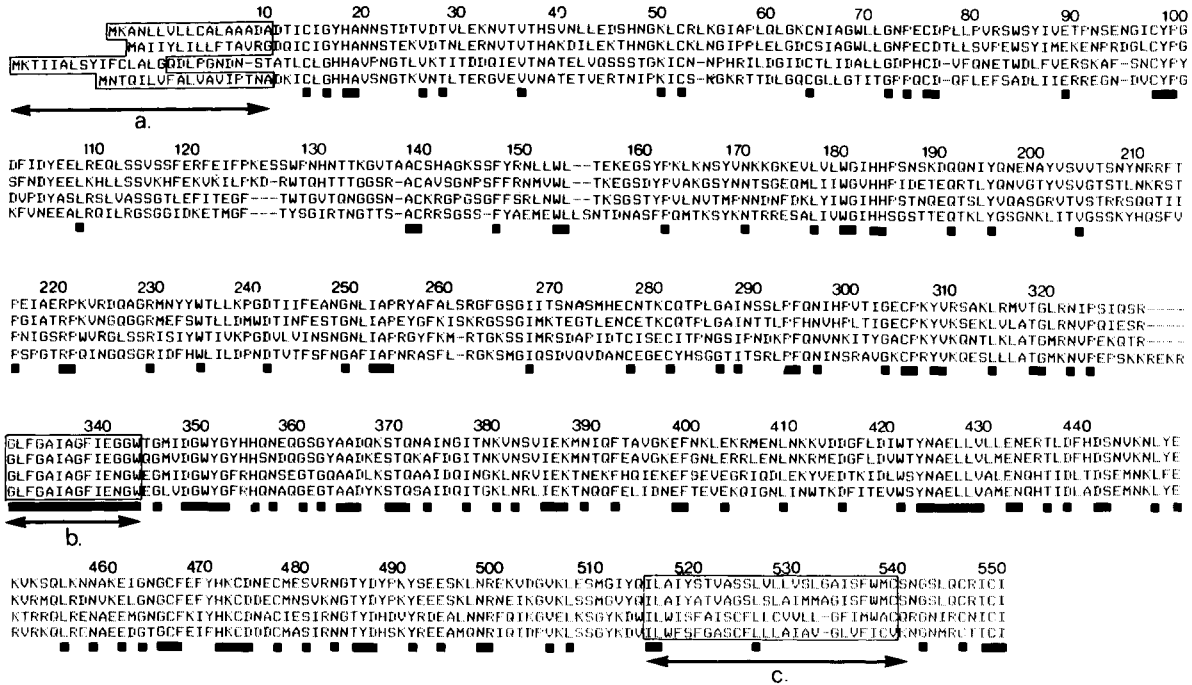

FIGURE 2. The amino acid sequences of the haemagglutinin of, from top to bottom, A/PR/8/34 (H1 subtype), A/Jap/305/57 (H2 subtype), A/Aichi/2/68 (H3 subtype) and A/FPV/Rostock/34 (H7 subtype) aligned to maximise amino acid homology. The numbering system is based on consecutive amino acid residues (ignoring gaps) starting at the N-terminus of the HA1 of the H3 subtype (A/Aichi/2/68). The boxed areas a, b and c are, respectively, the signal peptide, the conserved N-terminus of HA1 and the C-terminal hydrophobic tail. (From ref 37 with permission.)

TABLE 1. % Amino acid Conservation in different H subtypes

	H2	H3	H7
H1	58(79)	35(53)	33(51)
H2		36(50)	35(53)
H3			36(65)

The conservation in the HA1 subunit is given first, followed in brackets by the conservation in the HA2 subunit. See Fig 1 for the specific strains used in the comparison.

estimated from the extent of drift in the H3 series A/NT/60/68, A/Memphis/102/72 and A/Victoria/2/75 (32,9). Thus in theory, if this rate of drift were maintained and were also applicable to the H1 subtype, the 42% difference in the amino acid sequence of the HA1 subunit of the H1 and H2 subtypes could have arisen assuming the H2 subtype evolved from the H1 subtype in a 42 year period. But the surveillance of human influenza strains since their isolation in 1933 (3) suggests that the H1 subtype did not undergo such an extensive "drift" at least in Man. No intermediate linking strains spanning the H1 and H2 subtypes were isolated. Rather the H2 subtype appeared suddenly in 1957 (along with a new neuraminidase subtype) presumably from an animal or bird reservoir where influenza is common (33). There remains the possibility that drift of the H1 subtype giving rise to the H2 subtype occurred in an animal or avian reservoir. Indeed H1 strains do infect pigs (3) but again no evidence of intermediate strains between the H1 and H2 subtype have been observed in the last 48 years.

We therefore suggest that the H1 and H2 subtypes are evolutionarily more closely related to one another than either is to the H3 and H7 subtypes but that the evolutionary time scale cannot be estimated at present. It will be interesting to sequence fully some of the 8 other known animal or avian haemagglutinin molecules since other similarities between subtypes could have remained undetected in the traditional immunological screening methods for classifying haemagglutinin subtypes (2).

A shorter account of this work is in press elsewhere (37). An independent sequence analysis of the haemagglutinin gene of another H1 subtype, A/WSN/33, also shows homology with the H2 subtype (34).

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REFERENCES

1. Tyrrell, D.A.J. and Smith, J.W.G. Brit.med.Bull. 35, 77-85 (1979).
2. Schild, G.C. et al In "Structure and Variation in Influenza Virus" (eds. Laver, W.G. and Air, G.M.) 373-383 (Elsevier, New York, 1980).
3. Pereira, M.S. Brit.med.Bull. 35, 9-14 (1979).
4. Porter, A.G. et al Nature 282, 471-477 (1979).
5. Gething, M-J. et al Nature 287, 301-306 (1980).
6. Both, G.W. and Sleigh, M.J. Nucleic Acids Res. 8, 2561-2575 (1980).
7. Min Jou, M. et al Cell 19, 683-696 (1980).
8. Sleigh, M.J. et al In "Structure and Variation in Influenza Virus" (eds. Laver, W.G. and Air, G.M.) 69-78 (Elsevier, New York, 1980).
9. Verhoeyen, M. et al Nature 286, 771-776 (1980).
10. Ward, C.W. and Dopheide, T. Virology 103, 37-53 (1980).
11. Sanger, F., Nicklen, S. and Coulson, A.R. Proc.natn. Acad.Sci. USA 74, 5463-5467 (1977).
12. Sanger, F. et al J.molec.Biol. 143, 161-178 (1980).
13. Winter, G. and Fields, S. Nucleic Acids Res. 8, 1965-1974 (1980).
14. Fields, S., Winter, G. and Brownlee, G.G. Nature 290, 213-217 (1981).
15. Winter, G. et al Nucleic Acids Res. 9, 237-245 (1981).
16. Gronenborn, B. and Messing, J. Nature 272, 375-377 (1978).
17. Rothstein, R.J. et al In Methods in Enzymology (eds. Colowick, S.P. and Kaplan, N.C.) 68, 101-110 (Academic Press, London, 1979).
18. Messing, J., Crea, R. and Seeburg, P.H. Nucleic Acids Res. 9, 309-321 (1981).
19. Staden, R. Nucleic Acids Res. 8, 3673-3694 (1980).

20. Air, G.M. In "Structure and Variation in Influenza Virus" (eds. Laver, W.G. and Air, G.M.) 135-146 Elsevier, New York, 1980).
21. Sleigh, M.J., Both, G.W. and Brownlee, G.G. Nucleic Acids Res. 6, 1309-1321 (1979).
22. Lai, C.J. et al Proc. natn. Acad. Sci. USA 77, 210-214 (1980).
23. Robertson, J.S. Nucleic Acids Res. 6, 3745-3757 (1979).
24. Lamb, R.A. and Lai, C.J. Cell 21, 475-485 (1980).
25. Brownlee, G.G. In "Structure and Variation in Influenza Virus" (eds. Laver, W.G. and Air, G.M.) 385-390 (Elsevier, New York, 1980).
26. Waterfield, M.D. et al Brit. med. Bull. 35, 57-63 (1979)
27. Waterfield, M., Scrace, G. and Skehel, J. Nature 289, 422-424 (1981).
28. Ward, C.W. and Dopheide, T.A. Brit. med. Bull. 35, 51-56 (1979).
29. Nakamura, K., Bhown, A.S. and Compans, R.W. Virology 107, 208-221 (1980).
30. Laver, W.G., Gerhard, W., Webster, R.G., Frankel, M.E. and Air, G.M. Proc.natn.Acad.Sci.USA 76, 1425-1429 (1979).
31. Wiley, D.C., Wilson, I.A. and Skehel, J.J. Nature 289, 373-378 (1981).
32. Brownlee, G.G. In "Expression of Eucaryotic, Viral and Cellular Genes" (Academic Press, London) (in the press)
33. Laver, W.G. and Webster, R.G. Brit.med.Bull. 35, 29-33 (1979).
34. Hiti, A.L., Davis, A.R. and Nayak, D.P. Virology, in the press.
35. Hamlyn, P.H. et al Cell 15, 1067-1075 (1978).
36. Simoncsitz, A. et al Nature 269, 833-836 (1977).
37. Winter, G., Fields, S. and Brownlee, G.G. Nature (in the press).

DEFECTIVE INTERFERING INFLUENZA VIRUSES: COMPLETE SEQUENCE OF A DI RNA

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ABSTRACT

Influenza defective interfering (DI) viruses are produced by high multiplicity passages. They require the helper function of infectious viruses for their replication but in turn interfere with the replication of infectious virus. They contain smaller RNA segments not required for the replication of infectious viruses. These RNA segments (DI RNA) are responsible for the interference of homologous infectious virus. Influenza DI RNA segments are characteristic of a DI virus population and are usually produced from one of the three polymerase genes. DI RNAs appear to arise by internal deletion of progenitor RNAs in which both 3' and 5' ends are preserved. The fine structure of one of the DI RNAs (DI L3) has been deduced by recombinant DNA cloning and sequencing. The sequence of DI L3 has been compared to the sequence of its progenitor RNA (P1 polymerase gene) and the site of deletion has been located. DI L3 RNA is 441 nucleotides long. The 3' end of DI L3 RNA is identical to the first 197 nucleotides from the 3' end of P1 RNA. Thereafter the sequence of the P1 gene diverges because of a deletion of approximately 2050 nucleotide sequence. Finally, the last 244 nucleotides at the 5' end of DI L3 is identical to the 5' end of P1 RNA. DI L3 RNA contains both 5' and 3' termini including transcription stop, poly A addition signals. Furthermore the complemen-

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tary strand of DI RNA appears to contain an open reading frame 101 amino acids beginning with a codon for methionine at 25-27 nucleotides and terminating at the stop codon (pos. 328-330). The sequence at the deletion does not resemble that of spliced mRNAs and suggests the possible involvement of a replicational event rather than splicing in the formation of DI influenza RNAs.

INTRODUCTION

When animal viruses are serially passaged at high multiplicity defective interfering (DI) virus particles which need the helper function of homologous infectious virus but interferes with the replication of homologous infectious virus are generated. These particles are also referred to as von Magnus virus (or von Magnus particles) since von Magnus first demonstrated these particles by passing influenza viruses serially undiluted in 10-11 day old embryonated chicken eggs. Subsequently the observation of von Magnus has been confirmed in almost all cell virus systems which have been carefully analyzed (1). We have been studying DI influenza virus over the last 10 years and some of these findings have been reviewed recently (2). Here we shall discuss some of the common questions often raised in studying influenza DI virus and present the complete sequence of a DI RNA (DI L3) which has been deduced by using recombinant DNA technology.

Are These Noninfectious Influenza Virus Particles Produced at High Multiplicity Passages Truly Defective Interfering Particles?

Since influenza virus genome is segmented (3) it is possible to generate noninfectious particles if one or more of the viral segment is missing. However, unlike DI viruses, such virus particles although noninfectious would not be interfering. Therefore, defective virus particles produced at high multiplicity passages were tested for interference by coinfecting cells with both defective and infectious viruses. The results show that indeed these noninfectious particles were also interfering since they reduced the yield of infectious virus by more than 99% without a similar

effect on the particle production (4). Furthermore, although obtained from a temperature sensitive mutant, these particles can interfere at both permissive and nonpermissive temperatures with wild type virus and at permissive temperature sensitive mutants. Similarly DI virus could be produced from wild type virus and was equally interfering. Therefore these influenza von Magnus particles produced by high multiplicity passages are also defective interfering virus (DI virus). In addition to this yield reduction assay, a direct interference assay (see below) also demonstrated that these particles are also capable of interfering with the replication of infectious virus.

How are DI Influenza Viruses Quantified?

A reduction in the ratio of infectivity to virus particles (e.g. PFU/HA) has been commonly used as an indication of DI virus production. We have devised a more sensitive assay for measuring interference directly (5). In this procedure cells were infected with both DI virus at varying multiplicity and coinfected with a known amount of standard virus (e.g. 4 PFU/cell). Infectious centers produced by coinfected cells were determined and the multiplicity of DI virus was calculated by based on the Poisson distribution, $P(0) = e^{-m}$ or $m = \ln P(0)$, where m is the multiplicity of DI virus and P(0) is the fraction of cells not receiving DI virus as determined from the infectious center. These results showed that indeed DI virus reduces visible plaque formation by infectious virus and can be measured directly as defective interfering unit (DIU) similar to the sensitive assay of plaque forming unit (PFU). However, the lack of visible infectious center does not indicate complete inhibition of virus production but suggests that DI viruses cause a reduction in cytopathic effect.

How Many DI Virus Particles are Required for Interference?

Both assays (one using the reduction of infectious virus in the yield and the other using the reduction of infectious centers in the direct DIU assay) suggest that a single DI particle is capable of interfering infectious virus replication in a coinfected cell (5).

What is the Nature of Influenza DI Viral Genome?

Influenza DI virus, produced at high multiplicity serial passages have been shown to contain less RNP and RNA (6, 7) compared to that present in standard viruses. When labeled RNA from preparations containing influenza DI virus was analyzed by gel electrophoresis (PAGE) we found that: (a) in some DI virus preparations one of the P genes appeared missing or greatly reduced as has been reported previously (8-10); (b) in others there appears to be an increased reduction of larger RNA segments without any apparent reduction of a specific RNA segment (5, 11); (c) however, in all cases one or more small novel RNA segments were present in DI virus preparation (4, 5, 12, 23). These smaller RNA segments were called DI RNA segments since they appeared responsible for interference (see below). We have therefore concluded that the loss of larger RNA segments was the sequel and not the cause of interference. Since, in many preparations the loss of specific RNA segments could not account for the drastic loss of infectivity as has been reported earlier (11), we have proposed that the loss of RNA segments among the DI virus particles could be random rather than specific and further proposed that the presence of DI RNA would prevent multiplicity dependent reactivation of infectious particles using plaque assay (4). Indeed partial reactivation of virus functions at higher multiplicity of DI viruses has been obtained and would support our hypothesis (13).

DI RNA segments are characteristic of a DI virus preparation. Separate clones free from DI viruses would produce a unique set of DI RNA segments which once produced, would replicate in the presence of infectious virus and become the predominate species of DI RNAs in the virus preparation. However, after many passages DI RNA population may undergo alteration with smaller DI RNA segments replacing larger DI RNA segments.

How Common are These Influenza DI RNAs?

DI influenza viruses appeared extremely common and present in many stock virus preparations even after repeated cloning. Some clones contained DI viruses which could be demonstrated by amplification. Similarly almost all clonal stocks appear to contain small amounts of DI virus. DI virus is also common to many if not all influenza virus stocks that we have examined to date (14). Whether these DI viruses are only produced in cell culture and embryonated

eggs or are also present in animals during natural infection cannot be answered directly. Although we have no direct evidence, it is highly unlikely that influenza DI virus is not produced in natural infection. More likely DI virus is produced in animals but is not amplified because of the low multiplicity of virus particles in natural infection.

What is the Effect of Different Host Cells on Influenza DI Virus Production?

Since influenza viruses vary greatly in their susceptibility to host cells, we have analyzed the role of MDBK, CEF and HeLa cells on the DI virus production (14). We have found that the amount of influenza DI virus varied quantitatively in different host cells. In general cells that produced the largest amount of infectious virus also produced the maximum amount of DI virus. Thus MDBK cells produced maximum amount of DI virus, CEF cells intermediate and HeLa cells least amount of DI virus. However, the nature of DI RNAs produced in these three types of cells as judged by PAGE analysis and oligonucleotide mapping were essentially identical. Therefore, none of these cells produced a great selective pressure in the amplification of a specific influenza DI RNA segment over another DI RNA segment.

Can Influenza DI Virus Interfere Against Different Subtype Viruses?

Influenza DI viruses prepared from WSN virus (H1N1) was able to interfere with equal efficiency against WSN (H1N1), USSR (H1N1), Port Chalmers (H3N2), and Victoria (H3N2) viruses but not against influenza B virus (14) or other heterologous viruses (e.g. VSV, NDV). Therefore, DI virus of one influenza A subtype can interfere equally with viruses belonging to other influenza A subtypes. Gel analysis of DI RNAs showed that WSN DI RNA replicated equally well with the helper function of other subtype A viruses. Similarly DI RNAs of USSR and Port Chalmers viruses also replicated in the presence of other type A viruses. These results suggest that gene products required for the replication of DI RNAs as well as for interference can function equally well among viral subtypes. However, since DI influenza virus cannot interfere against other heterologous viruses as is the case with most DI virus mediated interference, interferon does not appear to play a major role in this system.

Are These DI RNAs Responsible for Interference?

Since influenza DI viruses, unlike other DI viruses, e.g. VSV DI virus or Sendai DI virus, contain, in addition to the DI RNA segments, other vRNA segments present in standard virus it became important to determine which of these RNA segments was responsible for interference. Four types of experiments were done to determine the role of specific RNA segments in interference. (a) By repeated cloning DI RNAs could be eliminated from infectious virus preparations. This would support the idea that influenza genome consists of 8 distinct RNA segments and that DI RNAs are not required for infectious cycle (3, 15). (b) During high multiplicity passages of infectious virus in either MDBK, HeLa or CEF cells, DI RNAs consistently appeared in the progeny particles and became amplified in subsequent high multiplicity passages. The appearance and the amount of DI RNAs correlated fairly well with the interfering activity (DIU/ml) present in the DI virus preparation. (c) In order to compare the target size of infectivity and interfering ability of influenza virus, a DI virus preparation was treated with different doses of UV light and the inactivation kinetics of plaque forming and interfering ability were measured as a function of UV dose (4). The results showed that UV target size of PFU was 40 times more sensitive than that of its interfering ability suggesting that the UV target size of interfering molecules was 40 times smaller than that of infectivity. Indeed, in this DI preparation the major DI RNA was approximately 1/40th the size of the total viral genome. This would also suggest that the small RNA segments are responsible for interference. However, there are other interpretations of this experiment. For example, plaque formation involves multiple cycles of infection and may be inhibited due to secondary effects of UV irradiation and may not correspond to the target size. Furthermore, since the genomic content of individual DI virus is not known, the UV resistance may simply indicate the increased number of copies of DI RNAs per virus particle rather than its target size. (d) Finally, direct experimental evidence that the small RNAs present in DI virus preparations are the interfering molecule(s) was obtained by demonstrating that RNP complexes containing DI RNA(s) were interfering while RNP complexes containing only vRNA segment(s) were not interfering (16). In this experiment RNP complexes were isolated from infectious and DI virus preparations and analyzed in glycerol velocity gradients. RNP complexes enriched in P and HA genes (Class I), NA and NP genes (Class II), and M and NS genes (Class III) can be separated. RNA analysis of RNP complexes

showed that Class I RNP complexes were completely free from Class III RNP complexes. In the RNP preparation of the DI virus the Class III RNP complexes also contained DI RNA segments in addition to M and NS genes. Using the infectious center reduction assay, it was shown that none of the RNP complexes (Class I, II, or III) from infectious virus possessed any interfering activity, whereas only the Class III RNP complexes containing DI RNA(s) isolated from DI virus caused interference. Class I RNP complex of DI virus that did not contain DI RNAs, did not possess any interfering activity. Dilution experiments further suggested that a single DI RNP was capable of causing interference of plaque formation by an infectious virus. These results suggest strongly that DI RNAs which arise during high multiplicity passages and which can be amplified by coinfection with homologous infectious helper virus are responsible for interference in segmented negative strand virus replication.

What is the Origin of Influenza DI RNAs?

The origin and the structure of DI influenza virus has been studied by a number of ways, namely (1) hybridization, (2) oligonucleotide mapping, (3) direct sequencing of viral and DI RNAs, (4) DNA cloning and deducing sequence of DI RNAs and progenitor RNAs. Initial hybridization studies showed that influenza DI RNAs are of some polkrity as viral RNA. Unlike VSV and Sendai DI RNAs they do not contain any extensive complementarity at the ends and there is no detectable double stranded stem in the structure of influenza DI RNA. Subsequently individual DI and vRNA segments were isolated and analyzed by oligonucleotide finger printing (17). The results showed that all of the DI RNAs examined to date originate from one of the 3 polymerase genes (P1, P2 or P3). DI RNAs of varying length can arise from more than one P gene. DI RNAs arising from a single P gene contain some common oligonucleotides of the progenitor gene suggesting some region of the P gene is preserved and that DI RNAs are not formed randomly from any region of a P gene. However, oligonucleotides present in smaller DI RNAs were not always subsets of larger DI RNAs suggesting a different mechanism possibly internal deletion was occuring in the formation of influenza DI RNAs.

Direct sequence analysis of the 3' and 5' ends both DI RNAs, and progenitor genes were done (18, 19). Both 5' and 3' termini of the progenitor genes were found to be preserved in DI RNAs indicating influenza DI RNAs were

formed by internal deletion of P genes. To learn further about the detailed structure of DI RNAs and their relationship to progenitor RNAs, a number of DI RNAs and P genes were cloned, sequences were deduced and the point of deletion was located.

What Is the Complete Sequence of DI L3 RNA and Its Relationship To P1 (Progenitor) Gene?

We have recently cloned a number of influenza RNA segments. Furthermore we have deduced the complete nucleotide and amino acid sequence of hemagglutinin (HA) of WSN (H0N1) virus and expressed antigenic determinants of HA in a bacterial as well as in eukaryotic cells (20-22 and this volume). The strategy of cloning using synthesized dodecamer primers has been previously described (20). Using a similar approach clones of P1 gene and DI RNA were obtained. They were further characterized by hybridization, restriction mapping and sequenced by Maxam and Gilbert techniques (25). Fig. 1 shows the restriction map and sequence strategy of D2-8 (DI L3). The complete sequence of L3 DI RNA contains 441 nucleotides (Fig. 2). The entire sequence except the last 33 nucleotides from the 5' end vRNA was obtained from both D2-8 and D2-62 clones both of which also contained the complete 3' terminal sequences. The first 197 nucleotide sequence from the 3' termini of both DI L3 and P1 gene obtained independently from four separate clones are identical and complete. Both possess the dodecamer primer used in cDNA synthesis by reverse transcription. However, the sequence of the P1 gene diverges from that of D2-62 and D2-8 from pos. 198, Fig. 3.

The sequence of DI-L3 was completed by the primer extension of vRNA by reverse transcription using a Taq 1 to Alu 1 primer (pos. 321 to 386) uniquely labeled at the 5' end of the Taq 1 site (Fig. 2). A sequence of 32 nucleotide (pos. 410 to 441) was derived by this method to complete the 5' end of the DI RNA. This terminal sequence contained the last 13 nucleotides at the 5' end common to all vRNA of influenza A viruses. Finally the last 36 nucleotide sequence (including the common 13 nucleotide) was essentially identical to the sequence derived directly by sequencing 5' vRNA (17) except for some minor corrections The P1 clone (1-39b) used for sequencing was approximately 2200 nucleotides long. It is complete at the 3' end of vRNA and lacks approximately 300 nucleotides from the 5' end of vRNA. The sequence at the 3' end of P1 gene and

DI RNA is very similar to that of the partial sequence of the fowl plague P1 RNA (24). Fourteen N-terminal amino acids proposed for fowl plague virus are identical to those shown here for WSN virus. When compared to fowl plague virus the first 70 nucleotides at the 3' end of P1 vRNA of WSN virus contains 4 changes, all in the third position of the codon without affecting any amino acid residue. The last 66 nucleotide from the 5' termini of WSN P1 vRNA are essentially identical to fowl plague virus except for 3 nucleotides.

Previously we have determined the characteristic oligonucleotide spots of DI RNAs and their respective progenitor genes (18). Complete sequence analysis of DI-L3 permitted us to identify these oligonucleotide spots based on their migration in a two dimensional gel (26, 27). The sequence of these 6 characteristic T1 oligonucleotides and their location in the L3 sequence is shown in Table 1. The order of these spots in the P1 gene and in different DI RNAs of P1 gene origin is shown in Fig. 4. These oligonucleotides represent approximately 43% of the total nucleotide sequences in DI L3

TABLE 1

Nucleotide Sequence of T1 Oligonucleotides of DI L3 RNA

Oligonucleotide Spot Number[1]	Sequence (Number of Nucleotides)	Sequence No.
D1	5'GAACUCCUCUUUCUUUAUCCUUCCA3' (25)	344-320
G4	5'GCUAAAUUCACUAUUUUU3' (18)	408-391
H5	5'GUCCAUCUUCCCCCUUUCU3' (18)	191-174
I6	5'GAAUUUUUCAAAUAA3' (15)	221-207
I11	5'GACAUCCAUUCAAAU3' (15)	33-19
J15	5'GCCUCCACCAUACU3' (14)	274-261

[1]Oligonucleotide spots have been reported earlier (10)

and is, therefore, unusually high for the sequence representation in characteristic T1 oligonucleotides. It is to be noted that H5 and I6 oligonucleotides flank the deletion point at the 5' and 3' ends of vRNA respectively with only 16 nucleotides in the intervening sequence containing multiple G residues. The new oligonucleotide spot produced

at the junction would be too small for a characteristic spot. Direct sequence analysis of the junction point of other DI RNAs will be required to identify the nucleotide sequence of the new spot(s) found in some DI RNAs (18). Since these six oligonucleotide spots are common in all DI RNAs of P1 origin the majority if not the entire sequence of DI L3 (i.e. approximately 197 nucleotides from the 3' and 244 nucleotides from the 5' end of vRNA) are likely to be present in all DI RNAs of P1 origin. However, although unlikely smaller internal deletions without affecting T1 oligonucleotide in other DI RNAs cannot be ruled out.

The complete sequence of a defective interfering influenza viral RNA presented here confirms and extends our previous results (17, 18) that influenza DI RNAs arise by internal deletion. Although internal deletion appears to be the principal mechanism in the generation of DI RNAs of influenza viruses, a deletion of one end with a copyback mechanism appear to be primarily responsible for the formation of DI RNAs of VSV and Sendai vriuses (28, 29). Recently, however, DI RNAs of VSV have been described that also have internal deletions (30, 31) although these internal deletions in DI LT RNAs appear different because a relatively small amount of RNA from the 5' end of vRNA is

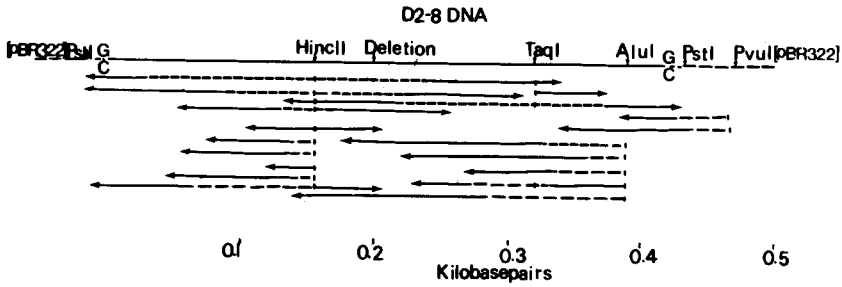

Figure 1. Restriction map and sequence strategy of D2-8 clone of DI L3 RNA. The 5' end of complementary DNA (+ strand) is on the left. G/C indicates the dG:dC tail at each end of the clone. Restriction sites used for ^{32}P labeling are Hinc II, Taq 1, Alu 1, in the insert and Pvu 1 in pBR 322 DNA. Solid arrows indicate the sequences read from individual gels and the broken line shows the nucleotide run off the gel.

```
                                                  20                                  40
Virion Strand (-)  3'  UCGCUUUCGUCCGUUUGGUAAACU  UAC CUA CAG UUA GGC UGA AAU
cRNA Strand    (+)  5'  AGCGAAAGCAGGCAAACCAUUUGA  AUG GAU GUC AAU CCG ACU UUA
                                                  Met Asp Val Asn Pro Thr Leu

                  60                                  80                                 100
GAA AAG AAU UUU CAC GGU CGU GUU UUA CGA UAU UCG UGU UGA AAG GGA AUA UGA CCU
CUU UUC UUA AAA GUG CCA GCA CAA AAT GCU AUA AGC ACA ACU UUC CCU UAU ACU GGA
Leu Phe Leu Lys Val Pro Ala Gln Asn Ala Ile Ser Thr Thr Phe Pro Tyr Thr Gly

                        120                                 140
CUG GGA GGA AUG UCG GUA CCC UGU CCU UGU CCU AUG UGG UAC CUA UGA ACG UUG UCC
GAC CCU CCU UAC AGC CAU GGG ACA GGA ACA GGA UAC ACC AUG GAU ACU GUC AAC AGG
Asp Pro Pro Tyr Ser His Gly Thr Gly Thr Gly Tyr Thr Met Asp Thr Val Asn Arg

160                         180                                 ↓200
UGU GUA GUC AUG AGU CUU UCC CCU UCU ACC UGU UGU UUA CGA CGU UGA AUA AAC UUU
ACA CAU CAG UAC UCA GAA AGG GGA AGA UGG ACA ACA AAU GCU GCA ACU UAU UUG AAA
Thr His Glu Tyr Ser Glu Arg Gly Arg Trp Thr Thr Asn Ala Ala Thr Tyr Leu Lys

   220                         240                                 260
UUA AGA AGG GGU CGU CAA GUA UGU CUU CUG GUC AGC CCU AUA GGU CAU ACC ACC UCC
AAU UCU UCC CCA GCA GUU CAU ACA GAA GAC CAG UCG GGA UAU CCA GUA UGG UGG AGG
Asn Ser Ser Pro Ala Val His Thr Glu Asp Glu Ser Gly Tyr Pro Val Trp Trp Arg

        280                         300                                 320
GAU ACC AAA GGU CUC GGG CUU AAC UAC GUG CUU AAC UAA AGC UUA GAC CUU CCU AUU
CUA UGG UUU CCA GAG CCC GAA UUG AUG CAC GAA UUG AUU UCG AAU CUG GAA GGA UAA
Leu Trp Phe Pro Glu Pro Glu Leu Met His Glu Leu Ile Ser Asn Leu Glu Gly Stop

         340                         360                 380                 400
UCUUUCUCCUCAAGUGACUCUAGUACUUCUAGACAAGGUGGUAACUUCUCGAGUCUGCCGUUUUUAUCACUUAAA
AGAAAGAGGAGUUCACUGAGAUCAUGAAGAUCUGUUCCACCAUUGAAGAGCUCAGACGGCAAAAAUAGUGAAUUU

              420
UCGAACAGGAAGUUCUUUUUUUCGGAACAAAGAUGA 5'
AGCUUGUCCUUCAAGAAAAAAAGCCUUGUUUCUACU 3'
```

Figure 2. Complete sequence of DI L3 RNA (both minus and plus strands) and the predicted amino acid sequence. DI L3 is 441 nucleotides long. Arrow (↓) shows the location of deletion between pos. 197 and 198. The predicted amino acid sequence of the amino-terminal end of P1 polymerase is also given. The amino acid sequence after the deletion point is underlined. Other two reading frames have multiple in phase stop codons.

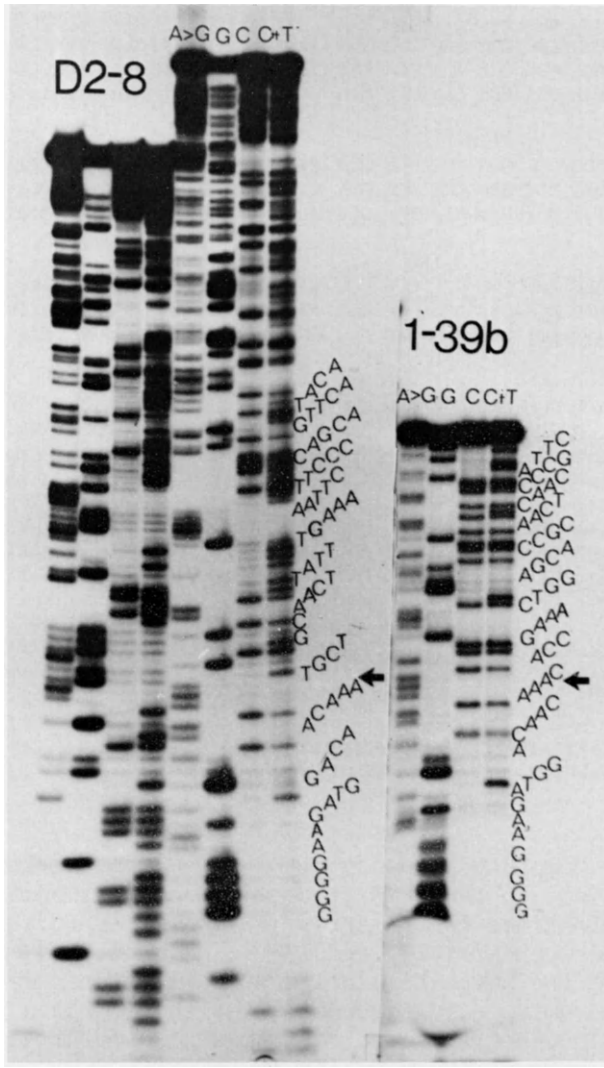

Figure 3. Sequencing gels of clones D2-8 (DI L3) and I-39b (P gene) showing the point of deletion. Both D2-8 and 1-39b clones were uniquely labeled with ^{32}P at the Hinc II position, processed through the sequencing reaction and analyzed in acrylamide gels (25). The sequence of both D2-8 and 1-39 were identical up to pos. 197 but was different thereafter.

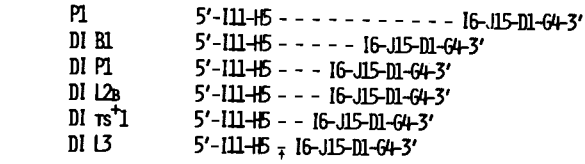

Figure 4. Order of T1 oligonucleotides of DI L3 RNA in P1 gene and in other DI RNAs of P1 origin. Characteristic T1 oligonucleotides have been reported previously (10). The sequence of T1 oligonucleotides and their position in the L3 RNA sequence are shown in Table 1.

preserved (30, 31), whereas in the influenza DI L3 RNA both ends are almost preserved equally.

The mechanism of RNA splicing does not appear to be involved in the formation of DI L3 RNA since the consensus splicing sites (32-34) are not present in either vRNA or cRNA of the P1 gene at the deletion point.

Based on the complete sequence analysis of DI L3 and the apparent conservation of a common nucleotide sequence among different DI RNAs of P1 origin a similar type of replicational or transcriptional event(s) appears to be responsible for the origin of DI RNA of influenza, VSV, or Sendai viruses. For example, as postulated for VSV and Sendai virus (28, 29), influenza polymerase with attached nascent chain may fall off the vRNA template but unlike VSV DI which copies back its own attached RNA, it will reinitiate polymerization downstream on the original template or the template of similar polarity. This may also explain the formation of two types of DI viruses (with or without internal deletion) in VSV. A similar mechanism involving strand switching by the polymerase has been proposed to explain the generation of complementary stem found in VSV DI LT2 RNA (35). A combination of termination, replication recognition sites and/or secondary structure of the template RNA may be responsible for the creation of diversity among the DI RNAs. A complete sequence analysis of a number of DI RNAs from P1, P2, or P3 genes and their progenitor genes will be needed to elucidate the mechanism of DI RNA function.

What is the Mechanism of Interference?

The function of influenza DI RNAs and the mechanism of interference is unknown at present. The majority of VSV DI RNA lacking the 3' end are unable to transcribe any functional mRNA (only approximately 46 nucleotide stem of VSV DI RNA is transcribed *in vivo* or *in vitro*) (36, 37). They presumably interfere with infectious virus replication by competition because of their reduced size and limited number of polymerase molecules. On the other hand, influenza DI L3 contains almost equal amount sequence from both 3' and 5' ends and therefore, unless prevented by a unique secondary RNA structure or termination signal, is expected to be transcribed like the progenitor virion RNA. DI RNA has also the U-rich termination stop signal (UUUUUUU, pos. 421-427) of P1 gene (38). Furthermore, DI mRNA if transcribed will possess structures similar to the expected structure of P1 mRNA including poly A addition site (AAAAAAA, pos. 421-427, ref. 38). Additionally since these sequences appear to be preserved in all DI RNAs of P1 origin, other DI RNAs will also have similar properties. Whether DI RNAs are capable of transcribing functional mRNAs remains to be seen.

What is the Role of Influenza DI Virus in the Evolution of Virus in Nature?

Whether DI virus exists in natural infection and if so, what is their role in virus evolution and viral pathogenesis are intriguing questions that are yet to be answered satisfactorily. As mentioned earlier that it is unlikely influenza DI virus like other DI virus is produced only in the laboratory. More likely, since influenza DI virus is produced in a variety of host cells, they are also produced in natural infection but are either not amplified since multiplicity of infection in natural transmission is extremely low or only partially amplified in the cluster of cells involved in forming the foci of infection. At present we do not have a sensitive assay to determine the presence of DI virus in a natural infection directly without passaging the infectious virus in cell cultures or embryonated eggs. If we assume that DI virus is also of frequent occurrence in nature as is found in the laboratory, they may also aid in the selection of natural influenza variants. Clearly other factors in addition to the immunological pressure are involved in the evolution of influenza virus variants since changes are not limited to HA and NA genes.

REFERENCES

1. Huang, A.S. and Baltimore, D. (1977). Comprehensive Virology 10,73-116.
2. Nayak, D.P. (1980). Ann. Rev. Microbiol. 34,619-644.
3. Palese, P. (1977). Cell 10,1-10.
4. Nayak, D.P., Tobita, K., Janda, J.M., Davis, A.R. and De, B.K. (1978). J. Virol. 28,375-86.
5. Janda, J.M., Davis, A.R., Nayak, D.P. and De, B.K. (1979). Virology 95,48-59.
6. Ada, G.L. and Perry, B.T. (1956). Gen. microbiol. 14,623.
7. Lenard, J. and Compans, R.W. (1975). Virology 65,418-26.
8. Choppin, P.W. and Pons, M.W. (1970). Virology 42,603-10.
9. Duesberg, P.H. (1968). Proc. Natl. Acad. Sci. USA 59,930-37.
10. Nayak, D.P. (1972). J. Gen Virol. 14,63-67.
11. Bean, W.J. Jr. and Simpson, R.W. (1976). J. Virol. 18,365-369.
12. Nakajima, F., Ueda, M. and Sugiura, A. (1979). J. Virol. 29,1142-1148.
13. Kavern, N., Kolomietz, L. and Rudneva, I. (1980). J. Virol. 34,506-511.
14. De, B.K. and Nayak, D.P. (1980). Virology 36,847-859.
15. Hay, A.J., Lomniczi, B., Bellamy, A. and Skehel, J.J. (1977). Virology 83,337-355.
16. Janda, J.M. and Nayak, D.P. (1979). J. Virol. 32,697-702.
17. Davis, A.R. and Nayak, D.P. (1979). Proc. Natl. Acad. Sci. USA 77,215-219.
18. Davis, A.R. and Nayak, D.P. (1979). Proc. Natl. Acad. Sci. USA 76,3092-3096.
19. Nayak, D.P., Davis, A.R. and De, B.K. (1981). In "Replication of Negative Strand Viruses" (D.H.L. Bishop and R.D. Compans, eds.) in press.
20. Davis, A.R., Hiti, A.L. and Nayak, D.P. (1980). Gene 10,205-218.
21. Hiti, A.L., Davis, A.R. and Nayak, D.P. (1981). Virology, in press.
22. Hiti, A.L., Davis, A.R. and Nayak, D.P. (1981). In "Replication of Negative Strand Viruses" (D.H.L. Bishop and R.D. Compans, eds.), in press.
23. Crumpton, W.M., Dimmock, N.J., Minor, P.D. and Avery, R.J. (1978). Virology 90,370-373.
24. Robertson, J.S. (1979). Nucl. Acid Res. 6,3745-3757.
25. Maxam, A. and Gilbert, W. (1977). Proc. Natl. Acad. Sci. USA 74,560-564.
26. Tu, C.D., Jay, E., Bahl, C.D. and Wu, G. (1976). Anal.

Biochem. 74,73-93.
27. Sanger, F., Brownlee, G.G., and Barell, B.G. (1965). J. Mol. Biol. 13,373-398.
28. Huang, A.S. (1977). Bacteriol. Review 41,811-821.
29. Kolakofsky, D. (1976). Cell 8,547-555.
30. Epistein, D.A., Herman, R.C., Chien, I. and Lazzarini, R.A. (1980). J. Virol. 33,818-829.
31. Perrault, J. and Semler, B.L. (1979). Proc. Natl. Acad. Sci. USA 76,6191-6195.
32. Rogers, J. and Wall, R. (1980). Proc. Natl. Acad. Sci. USA 77,1877-1879.
33. Sief, J., Khoury, G. and Dhar, R. (1979). Nucl. Acid Res. 6,3387-3398.
34. Lerner, M.D., Boyle, J.A., Mount, M.S., Wolin, L.S. and Steitz, J.A. (1980). Nature 283,220-224.
35. Keene, J.D., Piwnica-Worms, H. and Isaac, C.L. (1981). In "Replication of Negative Strand Viruses" (D.H.L. Bishop and R.D. Compans, eds.), in press.
36. Emerson, S.U., Dierks, P.M. and Parsons, T.J. (1977). J. Virol. 23,708-716.
37. Rao, D.D. and Huang, A.S. (1979). Proc. Natl. Acad. Sci. USA 76,3742-3745.
38. Robertson, J.S., Caton, A.J., Schubert, M. and Lazzarini, R.A. (1981). In "Replication of Negative Strand Viruses" (D.H.L. Bishop and R.D. Compans, eds.), in press.

THE INITIATION OF INFLUENZA VIRAL RNA TRANSCRIPTION BY CAPPED RNA PRIMERS

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I. ABSTRACT

RNAs containing a 5' terminal methylated cap structure (m^7GpppNm) strong stimulate (prime) influenza viral RNA transcription in vitro. The stimulation results not from hydrogen bonding between the capped RNA primer and the 3' end of the virion RNA but presumably from a specific interaction of the capped RNA primer with protein(s) in the transcriptase complex. On the basis of our data, we propose a mechanism for priming in which specific 5' terminal fragments are cleaved from the capped RNAs by a virion-associated endonuclease. These fragments would then serve as the actual primers for the initiation of transcription via the initial incorporation of a G residue at their 3' end. We show that virions and purified viral cores do contain a unique endonuclease that cleaves RNAs containing a 5' methylated cap structure preferentially at purine residues 10 to 13 nucleotides from the cap, generating fragments with 3' terminal hydroxyl groups. RNAs containing the 5' terminal structure GpppG could not be cleaved to produce these specific fragments. Consistent with our proposed mechanism, those capped fragments that actually function as primers could be linked to a G residue in transcriptase reactions containing (α-^{32}P)GTP as the only ribonucleoside triphosphate. The pattern of G and C incorporation onto these primer fragments strongly suggests that this incorporation is directed by the second and third bases at the 3' end of

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the virion RNA template, which has the sequence 3' UCG. Primer fragments with a 3' terminal A residue were more efficiently utilized than those with a 3' terminal G residue, indicating a preference for generating an AGC sequence in the viral mRNA complementary to the 3' end of the virion RNA. Cleavage of the RNA primer and initiation of transcription are not necessarily coupled, because a 5' fragment isolated from one reaction could be utilized as a primer when added to a second reaction. Uncapped ribopolymer inhibitors of viral RNA transcription inhibited the cleavage of capped RNAs. A similar priming mechanism occurs in vivo.

II. INTRODUCTION

Capped eukaryotic RNAs act as primers for the synthesis of influenza messenger RNA (mRNA) in vitro and transfer their 5' terminal methylated cap structure and a short stretch of nucleotides (about 10-15) to the viral mRNA (Bouloy et al, 1978; Plotch et al, 1979; Bouloy et al, 1979; Robertson et al, 1980). A similar process apparently also occurs in vivo because the viral mRNAs synthesized in the infected cell contain a short stretch of nucleotides at their 5' end, including the cap, that are not viral coded (Krug et al, 1979; Dhar et al, 1980; Caton and Robertson, 1980).

As an initial approach for determining the mechanism for this priming reaction, we previously identified those bases of a representative primer, β-globin mRNA, that were transferred to the viral RNA transcripts. Using ^{125}I-labeled globin mRNA as primer for in vitro transcription, we found that the predominant sequence at the 5' end of each viral mRNA segment was identical to the first 13 nucleotides (plus the cap at the 5' terminus of β-globin mRNA, which has the sequence: $m^7Gpppm^6A_1mC(m)ACUUGCUUUUGAC_{13}$.. (Lockard and RajBhandary, 1976). Because only the C residues were labeled with ^{125}I, these results indicated that either the first 12, 13 or 14 5' terminal bases of β-globin mRNA transferred to the viral mRNAs. Analysis of the minor ^{125}I-oligonucleotides found in the viral mRNAs indicated that shorter, 5' terminal fragments of β-globin (8-11, or 2-3 bases in length) were sometimes transferred and that the transferred pieces were most likely linked to G as the first base incorporated by the transcriptase. Evidence for G being the first base added during viral RNA transcription in vivo has also been obtained (Caton and Robertson, 1980).

The priming mechanism must involve recognition of the 5' terminal methylated cap structures (m^7GpppNm), because only RNAs containing such a cap are active as primers (Bouloy et al, 1978; Plotch et al, 1979; Bouloy et al, 1979; Bouloy et al, 1980). Removal of the m^7G of the cap by chemical or enzymatic treatment eliminates all priming activity, and this activity can be restored by enzymatically recapping the RNA (Plotch et al, 1979). The cap must contain methyl groups, since reovirus mRNAs with 5' GpppG ends were not active as primers (Bouloy et al, 1980). In fact, the absence of either methyl group greatly reduces priming activity (Bouloy et al, 1979). This is the only system in which the 2'-O-methyl group of the cap has been shown to have a strong and clear-cut effect on a specific function of a mRNA. Thus, the influenza virion transcriptase recognizes the entire m^7GpppNm cap structure, whereas only the terminal m^7G of the cap is apparently recognized in protein synthesis (Shatkin, 1976).

These and other experiments (to be discussed here) suggested to us a possible mechanism for priming, for which we will provide support here.

III. RESULTS AND DISCUSSION

1. Priming activity of an RNA does not require hydrogen-bonding with the template virion RNA. In terms of understanding the mechanism of priming, it was necessary to determine whether the priming RNA must contain a sequence complementary to the common 3' end (3'UCGU; Skehel and Hay, 1978; Robertson, 1979) of the virion RNA (vRNA) templates. In one approach to this problem, we determined whether 5' terminal fragments of natural mRNAs lacking such a complementary sequence were active as primers (Krug et al, 1981). Fragments of globin mRNA generated by partial alkali or ribonuclease T1 digestion were found to be effective primers. After fragmentation with alkali, however, there was about a 30% loss of total priming activity after fragmentation with mild alkali. This was almost certainly due to ring-opening of the terminal m^7G by alkali (Shatkin, 1976), because the fragments generated by ribonuclease T1 digestion were 4-8 fold more effective as primers on a molar basis than the intact globin mRNA. To determine the exact nucleotide length of the active 5' terminal fragments, globin mRNA and 2'-O-methylated alfalfa mosaic virus (AlMV) RNA 4 (each labeled with ^{32}P in the cap) were fragmented by partial alkali digestion, and the fragments of various chain lengths from each of these RNAs were resolved by gel electrophoresis and

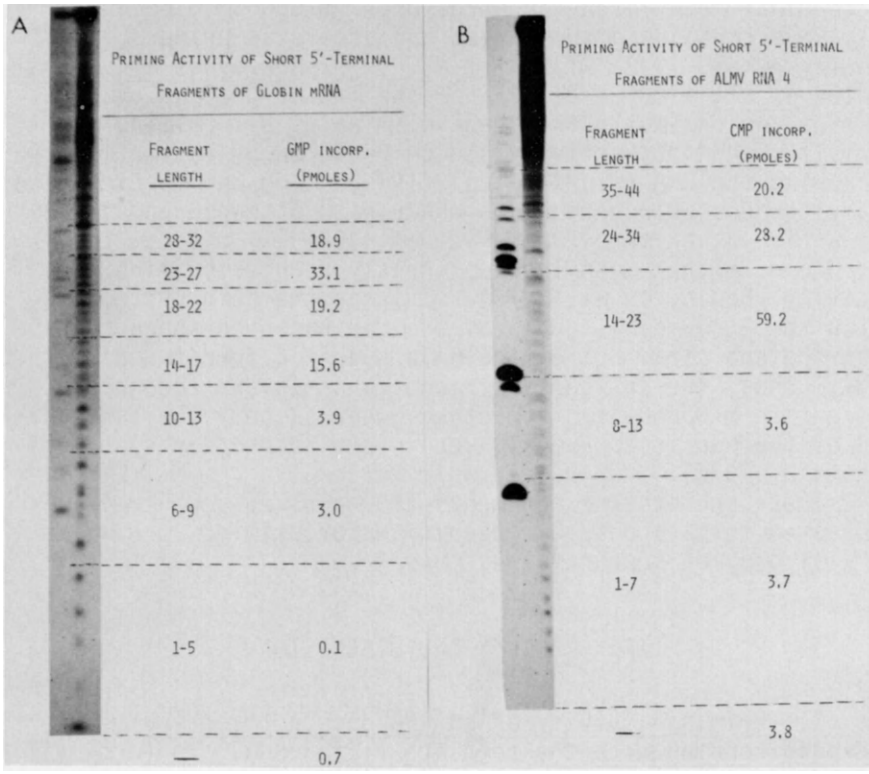

FIGURE 1. Priming activity associated with alkali-generated fragments of globin mRNA (A) and 2'-O-methylated AlMV RNA 4 (B). (A): A mixture of unlabeled and ^{32}P-cap-labeled globin mRNA was partially digested with mild alkali (50mM Na_2CO_3, PH8.5, 55 C, 30 min) and then subjected to electrophoresis on a 20% gel (right lane). The gel slices containing the indicated fragments were eluted, and each of the fragment preparations was assayed in a transcriptase reaction for 1 hr. at 31C with (8-^{3}H)GTP as labeled precursor. The left lane of the gel shows the partial T1 ribonuclease digest of the ^{32}P-labeled globin mRNA. (B): A mixture of unlabeled and ^{32}P-cap-labeled AlMV RNA 4 was partially digested with mild alkali and subjected to electrophoresis on a 20% gel (right lane). Each of the fragment preparations was assayed in a transcriptase reaction with (α-^{32}P)CTP as labeled precursor. The left lane shows the partial U2 ribonuclease digest of the ^{32}P-labeled AlMV RNA 4. From Krug et al (1981).

tested for priming activity (Figures 1A and 1B). With both these RNAs, 5' fragments as short as 14-23 nucleotides long were effective primers. In fact, with AlMV RNA 4, fragments of this size were more active per 5' termini than longer-size fragments. Since 5' terminal fragments 14-23 nucleotides long from either AlMV RNA 4 or β-globin mRNA do not contain a sequence complementary to the 3' end of influenza vRNA (Lockard and RajBhandary, 1976; Koper-Zwarthoff et al, 1977), the observed stimulation by these fragments cannot result from hydrogen-bonding between them and the 3' end of vRNA.

The same conclusion was obtained using capped ribopolymers as primers (Krug et al, 1980). Capped poly A and capped poly AU, neither of which contain a sequence complementary to the 3' end of vRNA, were about as effective primers as globin mRNA. Some, though lower, activity was seen with capped poly U, even though the 3' terminal 12-nucleotide sequence common to the eight influenza vRNA segments contain no A residues (Skehel and Hay, 1978; Robertson, 1979). Of the various polymers tested as primers, capped poly C was the least effective. Thus, while there is some effect of sequence on the efficiency of transcriptase priming by an exogenous capped ribopolymer, there is no requirement for the presence of a sequence complementary to the 3' end of the vRNA template.

2. Postulated mechanism for the priming of influenza viral RNA transcription. Based on our results, we postulated a mechanism for the priming of influenza viral RNA transcription by capped RNAs (Figure 2). The capped RNA is cleaved by a virion-associated nuclease to generate one or more 5' terminal fragments. In the example shown, β-globin mRNA is cleaved at the G residue 13 nucleotides from the 5' end. Some, or all, of these fragment(s) are the actual primers initiating transcription. In the absence of hydrogen-bonding between the primer and vRNA, the stimulation of initiation would result from a specific interaction between the capped RNA fragment and one or more proteins in the transcriptase complex. This specific interaction most probably requires recognition of the 5' terminal methylated cap structure of the primer, since priming activity requires the presence of this cap structure. As a result of this interaction, the transcriptase acquires the ability to initiate transcription and links a G residue to the 3' end of the 5' terminal fragment generated by the nuclease. It is most likely that the incorporation of the initial G residue is directed by the 3' penultimate C of the vRNA. Elongation of the viral RNA transcriptase would then follow. This mechanism predicts the existence of an intermediate in the priming reaction: 5' terminal fragment(s)

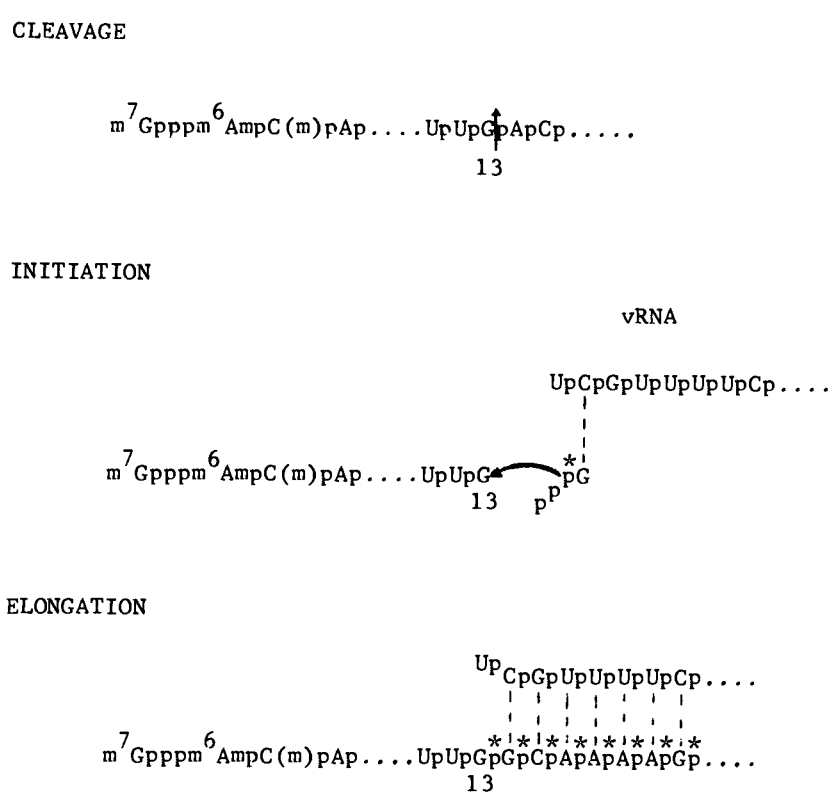

FIGURE 2. Postulated mechanism for the priming of influenza viral RNA transcription by β-globin mRNA and other RNAs. From Plotch et al (1981).

cleaved from the mRNA primer. In addition, in a reaction in which the only triphosphate present is GTP, those 5' terminal fragments that are the actual primers initiating transcription should be linked to a G residue. Here, we will identify both these species, thereby providing strong support for the postulated mechanism of priming.

3. Identification of the 5' terminal fragments that are the actual primers initiating transcription. Globin mRNA was incubated with detergent-treated virus in the presence of (α-^{32}P)GTP as the only ribonucleoside triphosphate, and the reaction products were analyzed by electrophoresis on 20% acrylamide gels containing 7M urea (Figure 3).

Two major labeled bands were observed in the size range of about 15 nucleotides. Sequence analysis of band 2 indicated that it was the fragment resulting from cleavage at the G13 residue of β-globin mRNA (Lockard and RajBhandary, 1976) to which one labeled G residue was added, i.e., $m^7Gpppm^6AmCACUUGCUUUUUGp^*G$. This is the species predicted
1 13
from the postulated mechanism. Thus, the 5' terminal fragment of β-globin mRNA cleaved at G13 can be presumed to be the actual primer that initiates influenza viral RNA transcription. Band 1 was the same G13 fragment of β-globin mRNA with two G residues added, indicating that more than one G can be added to the primer fragment in the absence of the other ribonucleoside triphosphates. No fragments of α-globin mRNA with 3' terminal labeled G residues were found, even when the same experiment was performed using purified α-globin mRNA.

When similar experiments were performed using either (α-^{32}P)ATP or (α-^{32}P)UTP as the only ribonucleoside triphosphate in the transcriptase reaction, no incorporation of label into fragments of globin mRNA was detected. With (α-^{32}P)CTP as the only ribonucleoside triphosphate, however, terminally labeled fragments of both β- and α-globin mRNA were found. The β-globin mRNA species was the same G13 fragment with a single C residue added, and the α-globin mRNA species was the cleavage product at nucleotide G10 (Baralle, 1977) to which one C residue was added, i.e., $m^7Gpppm^6AmCAUUCUGGp^*C$. These results suggested that the
1 10
G residue at the third position from the 3' end of vRNA (see Figure 2) most likely directed C incorporation in these reactions, with the G residue at the 3' end of the primer fragments of α- and β-globin mRNA lining up opposite the 3' penultimate C of the vRNA template. In the case of α-globin mRNA, the two G residues at the 3' end of its G10 fragment presumably hydrogen-bond to the UC at the 3' end of the vRNA (see Figure 2), thereby precluding G addition to the fragment and allowing only C addition.

Consequently, we would predict that primer fragments of mRNAs which contain any nucleotide other than G at their 3' ends should not be capable of lining up with the 3' penultimate C of the vRNA template and hence should not be terminally labeled in the presence of only (α-^{32}P)CTP. If, however, the penultimate C of the vRNA normally acts to direct the incorporation of the first base, a G residue (see Figure 2), then primer fragments containing nucleotides other than G at their 3' end should be labeled when incubated in the presence of only (α-^{32}P)GTP or in the presence

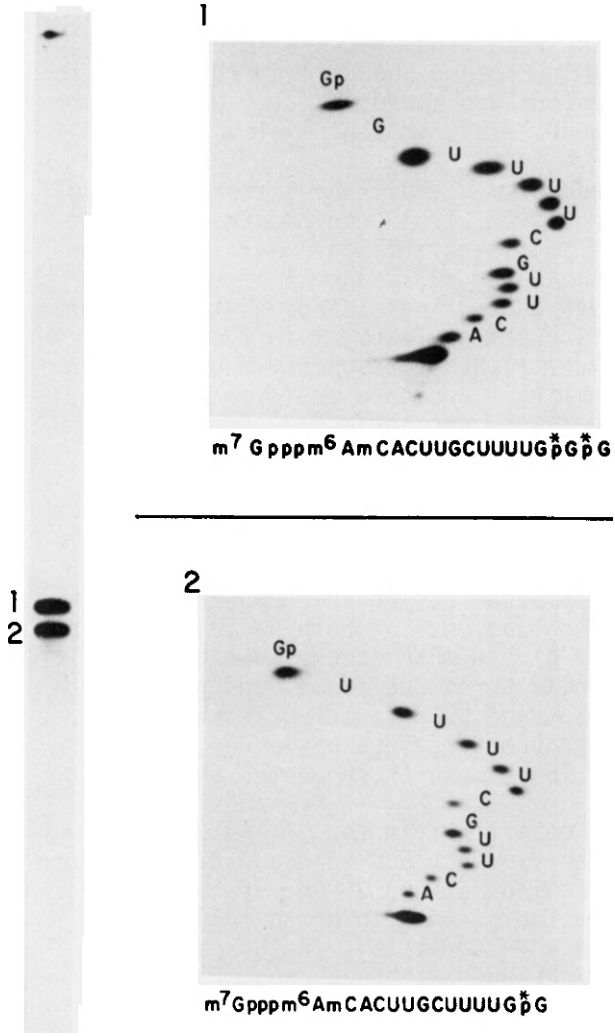

FIGURE 3. Identification and sequencing of the 5' terminal fragments of β-globin mRNA that initiate influenza viral RNA transcription. Globin mRNA was incubated with detergent-treated virus in the presence of (α-^{32}P)GTP, and the RNA products were analyzed on a 20% acrylamide gel in 7M urea. The RNA in the bands (1 and 2) was eluted, and after β-elimination, (Plotch et al, 1979) was partially digested with sodium bicarbonate. The digest was analyzed by two-dimensional gel electrophoresis. The identity of the 3' terminal nucleoside monophosphate (Gp in both RNAs 1 and 2) was determined by thin-layer chromatography of the RNase. The m^7Gpppm^{6}AmC sequence, which could not be directly determined, is based on the known cap structure of β-globin mRNA.

of both unlabeled GTP and (α-^{32}P)CTP. This was shown to be the case, using as primers capped poly A, capped poly AU, and AlMV RNA 4 (with the 5' terminal sequence m^7GpppGmUUUUUAUUUUUAAUUU...and no other G residue until position 39 from the 5' end (Koper-Zwarthoff et al, 1977). The primer fragments derived from these RNAs (i.e., the

fragments labeled in a transcriptase reaction in the presence of only (α-^{32}P)GTP contained totally, or predominantly, an A residue at their 3' end. With $m^7GpppGmC(AU)_n$, all the primer fragments contained 3' terminal A and not U, and with AlMV RNA 4, which has the extremely U-rich sequence at its 5' end noted above, the predominant primer fragment (75-80% of the time) was the cleavage product A13. The minor primer fragments of AlMV RNA 4 were the cleavage products at U11 and U12. Thus, the pattern of G and C incorporation onto the primer fragments derived from these mRNAs (A-terminated) and onto those derived from globin mRNA (G-terminated) indicated that this incorporation is almost certainly directed by the second and third bases at the 3' end of vRNA: C incorporation only occurred next to a G residue, which was either already present at the 3' end of the primer fragment or was put there by prior incorporation.

4. Identification of a cap-dependent endonuclease in influenza virions that cleaves capped RNAs at purine residues near the 5' terminus. In order to demonstrate the cleavage of a capped RNA directly, without G addition, several capped RNAs containing ^{32}P label only in their 5' terminal methylated cap structure $m^7G\overset{*}{p}ppNm$ were used as substrates in the absence of the four ribonucleoside triphosphates (Plotch et al, 1981). When cap-labeled AlMV RNA 4 was incubated with detergent-treated virus in the absence of ribonucleoside triphosphates, four capped fragments were predominantly generated (in the size range of the 11 to 15 nucleotide-long alkali-derived fragments of AlMV RNA 4) (Figure 4, lane 3). The major species, representing 75-80% of the total, was the cleavage product at A13, i.e., $m^7Gppp\underset{1}{G}mUUUUUA\underset{13}{U}UUUA$, and the minor cleavages were at U11, U12 and A14. The fact that the predominant cleavage in this extremely U-rich region was at an A residue reflects the strong preference by the virion endonuclease for cleavage at purines (see later). These cleavage products were shown to contain a 3'-hydroxyl group (Plotch et al, 1981), as expected for primer molecules, and thus they migrated slower than the alkali-derived marker fragments of the same size which contain a 2', 3' cyclic phosphate group.

The fact that the A13 fragment was utilized as primer could be verified by adding unlabeled GTP to the reaction containing the ^{32}P-cap-labeled AlMV RNA 4 (lanes 4 and 5) (Plotch et al, 1981). The A13 fragment disappeared and was replaced by more slowly migrating bands, the mobilities of which were consistent with the addition of one to three

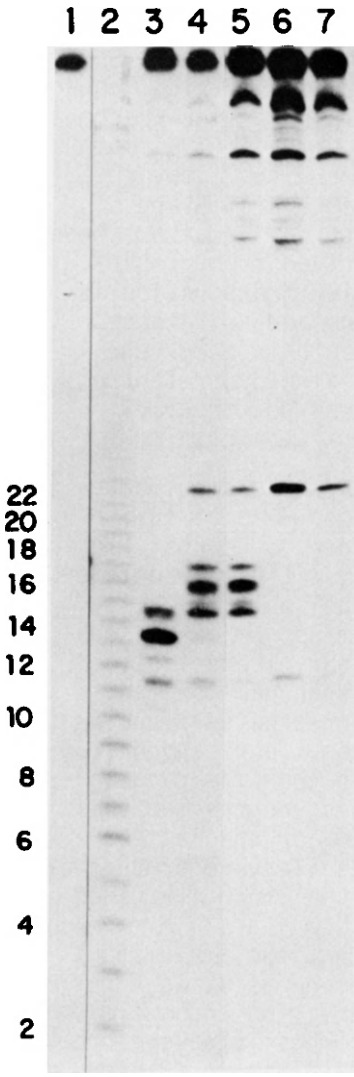

FIGURE 4. Identification of a cap-dependent endonuclease in influenza virions that cleaves AlMV RNA 4 at specific positions near its 5' end. AlMV RNA 4 containing a ^{32}P-labeled cap structure ($m^7G\overset{*}{p}pppGm$) was incubated at 31°C in the absence of virus (lane 1), or with detergent-treated virus in the absence of ribonucleoside triphosphates (lane 3) or in the presence of 25 μ M unlabeled GTP (lanes 4 and 5). AlMV RNA 4 containing a ^{32}P-labeled $G\overset{*}{p}ppG$ 5' end was incubated at 31°C with detergent-treated virus in the absence of ribonucleoside triphosphates (lane 6) or in the presence of 25 μ M unlabeled GTP (lane 7). The phenol-extracted RNA was analyzed by electrophoresis on 20% acrylamide gel in 7M urea. Lane 2 is the partial alkali digestion products of AlMV RNA 4 containing a $m^7G\overset{*}{p}pppGm$ cap, and the numbers on the left refer to the chain lengths of these products, counting the Gm residue as the first base. From Plotch et al (1981).

G-residues to the A13 fragment. The small amount of the U12 fragment generated by the nuclease also disappeared in the presence of GTP, suggesting that it was also used as a primer. No priming by the A14 fragment has been detected. This strongly suggests that the transcriptase can only effectively use as primers 5' terminal fragments 13 nucleotides long or less (plus the cap).

To determine whether specific cleavage of the primer mRNA is dependent on the presence of a methylated cap structure, AlMV RNA 4 containing a 5' terminal ^{32}P-labeled G$\overset{*}{p}$ppG blocking group was incubated with detergent-treated virus in the absence (lane 6) or presence (lane 7) of unlabeled GTP (Plotch et al, 1981). No primer fragments resulting from cleavage at U12, A13 or A14 were detected, and as a result, no bands resulting from the addition of G residues to these cleavage products were produced. A small amount of cleavage at U11 was seen, but no growth of this cleavage product occurred in the presence of GTP. Other, presumably non-specific cleavages, generating fragments that were not utilized to initiate transcription, occurred at about nucleotide 22 and at nucleotides further from the 5' end. Most of these cleavages were also seen with the AlMV RNA 4 containing a methylated cap structure. Thus, a methylated cap structure in an RNA is required by the viral endonuclease to generate those specific fragments that serve as primers for viral RNA transcription. This is the only known example of a cap-dependent endonuclease.

The endonuclease preferentially cleaves at purines (Plotch et al, 1981). This was seen most dramatically with BMV RNA 4. With this RNA (containing a ^{32}P-labeled methylated cap structure m^{7}G$\overset{*}{p}$ppGm), two major cleavages occurred (Figure 5A, lane 2): at nucleotide G12 (Dasgupta et al, 1975), i.e., m^{7}GpppGmUAUUAAUAAUG, and at nucleotide A10 (Plotch et al, 1981). No cleavage at U11, however, occurred. In addition, with both α- and β-globin mRNA, cleavage almost certainly occurred primarily, if not entirely, at G residues (G10 with α-globin mRNA and G13 with β-globin mRNA), and with the ribopolymer capped poly AU (1:1), cleavage occurred only at A residues and not at U residues. Even with AlMV RNA 4, which has the extremely U-rich 5' end noted above, cleavage still occurred preferentially at a purine, the A13 residue. However, with this RNA, some cleavage at the U residues at positions 11 and 12 (and at the A14 residue) also occurred, particularly when larger amounts of the virion endonuclease were used. The ability of the endonuclease to cleave occasionally at U residues would explain why the ribopolymer capped poly U exhibits some priming activity (Krug et al, 1980). Nevertheless, with most capped RNAs containing a relatively even distribution of purines and pyrimidines near their 5' end, cleavage most probably occurs almost exclusively at purines.

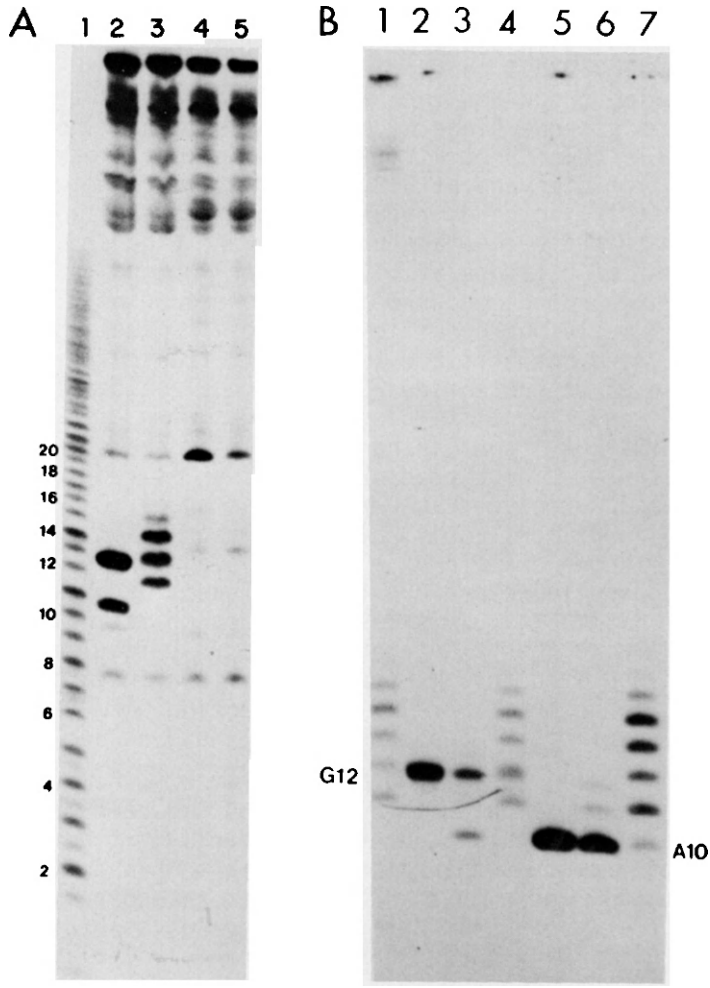

FIGURE 5. (A) Specific cleavage of BMV RNA 4 by the influenza virion endonuclease. BMV RNA 4 containing either a ^{32}P-labeled methylated cap structure m^{7}G$\overset{*}{p}$pGm (lanes 2 and 3) or a ^{32}P-labeled unmethylated cap structure G$\overset{*}{p}$pG (lanes 4 and 5) was incubated with detergent-treated virus in the absence of ribonucleoside triphosphates (lanes 2 and 4) or in the presence of 25 μ M unlabeled GTP (lanes 3 and 5). After incubation, the phenol-extracted RNA was analyzed by electrophoresis on a 20% acrylamide gel in 7M urea. Lane 1 is the partial alkali digestion product of BMV RNA 4 containing a m^{7}G$\overset{*}{p}$pGm cap, and the numbers on the left refer to the chain lengths of these products, counting the Gm residue as the first base. From Plotch et al (1981).

5. Capped fragments generated by cleavage at A residues are preferentially utilized as primers to initiate transcription. As noted above, BMV RNA 4 was cleaved at both nucleotides A10 and G12, with the latter cleavage usually occurring more frequently (Figure 5A, lane 2). However, little or no priming by the G12 fragment occurred, whereas the A10 fragment was an effective primer. Thus, when unlabeled BMV RNA 4 was incubated with detergent-treated virus in the presence of (α-^{32}P)GTP, the only primer fragment detected was the A10 cleavage product with one to three G residues added (Plotch et al, 1981). The utilization of the A10 fragment as primer could also be shown using ^{32}P-cap-labeled BMV RNA 4 as primer in the presence of unlabeled GTP (Figure 5A, lane 3): the A10 fragment disappeared, and three more slowly migrating major bands appeared. On the basis of this band pattern, it seemed likely that in the presence of GTP the G12 fragment was cleaved further to yield additional A10 fragment which was then used as primer. Thus, a band corresponding to the G12 fragment was not apparent, and the three bands observed contained more radioactivity than was present in the A10 fragment generated in the absence of GTP (lane 2).

To verify this conclusion, the A10 and G12 fragments were isolated from a nuclease reaction (done in the absence of GTP) and incubated in a subsequent reaction with detergent-treated virus in the absence (Figure 5B, lanes 3 and 6) and presence (lanes 4 and 7) of GTP (Plotch et al, 1981). The A10 fragment was not cleaved further by incubation with the virus in the absence of GTP (lane 6). The faint bands migrating more slowly than the A10 fragment were most probably the result of a low level of G addition to the A10 fragment, due to trace amounts of GTP present

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(B) Ability of fragments cleaved from BMV RNA 4 by the virion endonuclease to be utilized directly as primers for the initiation of transcription in a subsequent incubation with the virus. The fragments cleaved at the A10 residue and the G12 residue (Figure 5A, lane 2) was eluted from the gel and purified. The G12 (lanes 2-4) and A10 (lanes 5-7) fragments were then separately incubated in the absence of virus (lanes 2 and 5); or with detergent-treated virus in the absence of ribonucleoside triphosphates (lanes 3 and 6) or in the presence of 1.0 mM unlabeled GTP (lanes 4 and 7). Lane 1 shows the band pattern obtained when intact BMV RNA 4 containing a $m^7G\overset{*}{p}ppGm$ cap structure was incubated with detergent-treated virus in the presence of 1.0 mM unlabeled GTP. From Plotch et al (1981).

in the virus. In the presence of added GTP (lane 7), one to five G residues were linked to this primer fragment, yielding a band pattern nearly identical to that seen when intact cap-labeled BMV RNA 4 was incubated with virus in the presence of GTP (lane 1). This demonstrates that an exogenously added fragment of the proper size and 3' terminus can serve as primer directly without further modification and that consequently the utilization of an RNA fragment as a primer does not have to be directly coupled to the nuclease reaction that generates that fragment. In contrast to the A10 fragment, some of the G12 fragment was cleaved further in the absence of added GTP (lane 3), yielding the A10 fragment. In the presence of added GTP (lane 4), this cleavage was carried essentially to completion and one to five G residues were added to the resulting A10 fragment. Thus, the preference for the A10 fragment over the G12 fragment was so strong that the G12 fragment was not used directly as primer, but was first converted to the A10 fragment.

The preference for A-terminated fragments extended across different capped RNA species. Thus, the A-terminated fragments of AlMV RNA 4 and BMV RNA 4 were much more efficiently utilized for initiation (G-addition) than the G-terminated fragment of globin mRNA (Plotch et al, 1981). Preferential utilization of the A-terminated fragments was as much as 10-fold.

6. Inhibition of the cap-dependent endonuclease by uncapped ribopolymers. Influenza viral RNA transcription is effectively inhibited by certain uncapped ribopolymers with minimal secondary structure, with poly S^4U and poly AG being the most potent inhibitors (Krug et al, 1980; Smith et al, 1980). These two ribopolymers were found to inhibit almost completely the specific cleavage of cap-labeled ($m^7G\hat{p}ppGm$) AlMV RNA 4 catalyzed by purified viral cores (Plotch et al, 1981), whereas poly A, which has been shown not to inhibit transcription (Krug et al, 1980; Smith et al, 1980), did not inhibit the cleavage reaction (Plotch et al, 1981). Thus, the ribopolymer inhibitors of viral RNA transcription inhibit the specific cleavage of capped RNAs.

IV. DISCUSSION

Our results have shown that the priming of influenza viral RNA transcription by capped RNAs occurs as shown in Figure 6. An endonuclease associated with the transcriptase complex cleaves the capped RNA predominantly, if not

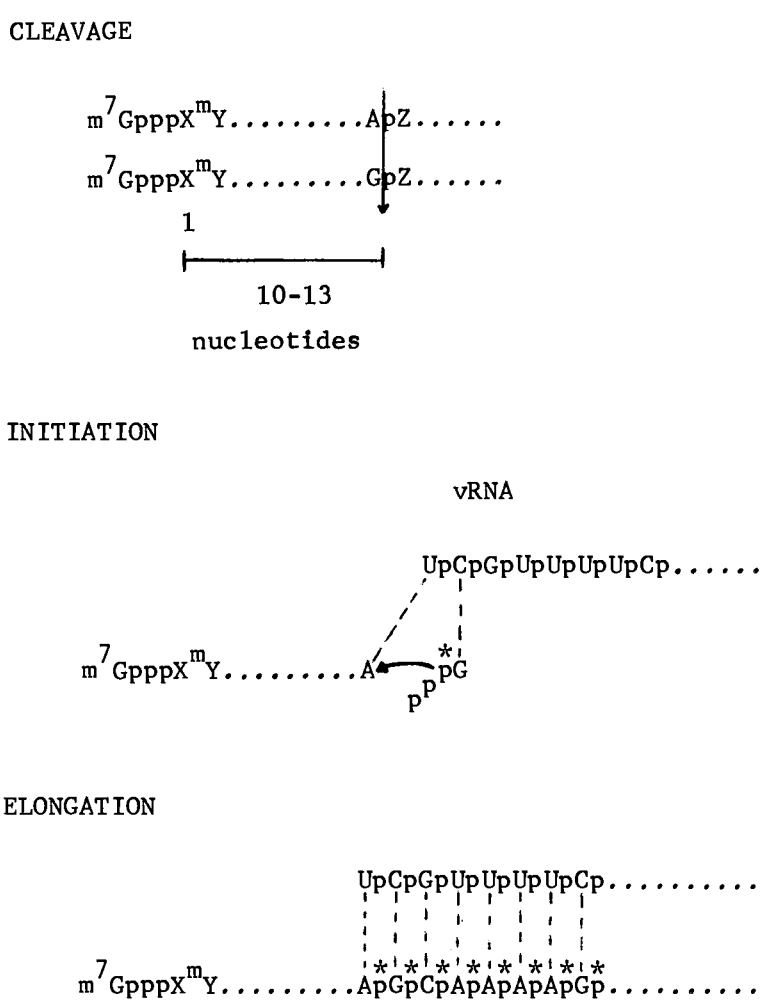

FIGURE 6. Mechanism for the priming of influenza viral RNA transcription by capped RNAs.

entirely, at a purine residue, A or G, 10 to 13 nucleotides from the 5' cap. These specific cleavages require the presence of a methylated cap structure in the RNA substrate. After the specific cleavage, the transcriptase preferentially utilizes A-terminated fragments as primers to initiate transcription. Transcription is initiated by the incorpor-

ation of a G residue onto the capped primer fragment. This G incorporation is almost certainly directed by the 3' penultimate C of the vRNA template. The cleavage and initiation steps are not necessarily coupled, because an appropriate nuclease-generated fragment isolated from one reaction can without any apparent modification directly initiate transcription when added to a second reaction.

As a consequence of the preference for the utilization of A-terminated over G-terminated fragments as primers, an AGC sequence would preferentially be generated in the viral mRNA, complementary to the UCG sequence at the 3' end of the vRNA. Recent experiments indicate that this also occurs during *in vivo* viral RNA transcription primed by cellular capped RNAs. The NS (nonstructural) segment of WSN virus was cloned in pBR322, and an appropriate restriction fragment derived from this cloned DNA was used as primer for the reverse transcriptase-catalyzed entension of the *in vivo* viral mRNA coding for NS_1: an A was found to be the predominant nucleotide opposite the 3' terminal U of the vRNA (Beaton and Krug, manuscript in preparation). A similar finding for the NS_2 mRNA has been reported (Lamb and Lai, 1980). Thus, the specificities of the virion endonuclease and transcriptase observed *in vitro* also occurs *in vivo*. In addition, because of the presence of an A at this position, it is conceivable that the same initiation step could be used for the synthesis of both viral mRNA and the full-length transcripts that are the putative templates for vRNA synthesis (Hay *et al*, 1977). Generation of the full-length transcripts would then involve removal of the host-derived sequence by nucleolytic cleavage at the 5' side of the A residue, thereby leaving these transcripts with the proper 5' AGC...sequence. Alternatively, the virus-coded protein(s) required for the synthesis of these full-length transcripts (Hay *et al*, 1977) could enable the transcriptase to initiate RNA synthesis without a primer at the 3' terminal U (instead of at the 3' penultimate C as occurs during viral mRNA synthesis).

The 5' methylated cap structure is recognized at the cleavage step of the reaction. In fact, as is the case with the complete transcription reaction, each of the methyl groups of the cap are apparently recognized. Thus, the cleavage of a cap-labeled RNA has been found to be inhibited by an excess of an unlabeled RNA containing a fully methylated cap structure (m^7GpppNm) but not by an excess of an unlabeled RNA containing a cap lacking a 2'-O-methyl group (m^7GpppN) (Ulmanen, Broni and Krug, manuscript in preparation). It was expected that the cap would also be recognized at the initiation step, where it would

mediate the specific interaction with one or more transcriptase proteins that causes the stimulation of initiation. This expectation has recently been verified. The 5' terminal A13 fragment of AlMV RNA 4 with a methylated cap structure (m^7GpppGm) was shown to act as a primer directly without prior modification, whereas the same fragment with a GpppG blocking group was inactive (Ulmanen, Broni and Krug, manuscript in preparation).

All the steps shown in Figure 6 are carried out by purified viral cores which contain four known viral proteins (Plotch et al, 1981): the nucleocapsid protein (NP) (60,000 daltons) and the three P(P1, P2, P3) proteins (85-90,000 daltons) (Rochovansky, 1976; Inglis et al, 1976). The NP protein, the predominant viral protein in the core, probably has primarily a structural role, as it is situated along the vRNA chains at approximately 20 nucleotide intervals (Compans et al, 1972). The P proteins, representing only about 8 percent of the total protein in cores (Inglis et al, 1976), are most likely the proteins which catalyze transcription. Studies with temperature-sensitive mutants of the WSN influenza A strain indicate that P1 and P3 are required for transcription and that P2 is also involved in viral RNA synthesis (Krug et al, 1975; Palese et al, 1977). One or more of the P proteins can be presumed to be the cap-dependent nuclease, and one or more the transcriptase. It will be of great interest to establish which P protein(s) carry out these functions and which also recognize methylated cap structures, and to compare this viral cap-recognizing protein(s) to the cellular cap-recognizing protein (24K protein) associated with ribosomes) (Sonenberg et al, 1979).

The virion endonuclease was effectively inhibited by poly S^4U and poly AG, consistent with these two inhibitors acting at the first step in mRNA-primed transcription. However, these ribopolymers also inhibit viral RNA transcription primed by the dinucleotide ApG (Krug et al, 1980; Smith et al, 1980), which does not require the action of the endonuclease, indicating that elongation is also apparently inhibited. A plausible explanation for these results would be that the endonuclease and other transcriptase proteins are tightly associated in a complex so that inhibition of one component also inhibits the other components. Thus, it is not yet certain that poly S^4U and poly AG act directly on the endonuclease protein.

Though a cap-dependent endonuclease such as found in influenza virions has not been described in any other viral or cellular system, it is reasonable to ask whether a similar activity can be expected to be found in other

systems. Several possibilities can be suggested. For example, cap recognition may be critical for the step in the processing of heterogeneous nuclear RNA in which the RNA is cleaved near its 5' terminus to produce fragments (albeit longer than those generated by the influenza virion enzyme) which are subsequently ligated onto 3' distal regions of the transcript (Darnell, 1978). Another possible role for a cap-dependent cellular endonuclease may be in mRNA turnover. Since the cap makes mRNAs more resistant to cellular nucleases (Furuichi et al, 1977a; Shimotohno et al, 1977), the initial removal of the capped 5' end of a mRNA by a cap-dependent endonuclease would facilitate subsequent RNA degradation. Recent evidence suggests that mRNA turnover may be important in the regulation of the expression of some genes in eukaryotes (Nevins and Winkler, 1980).

If, however, a cap-recognizing endonuclease were found to be unique to influenza virions, then it might be an ideal target for specific anti-influenza virus drugs, possibly related to the uncapped ribopolymers inhibitors described above. Further investigation of the mechanism of action of this nuclease would therefore be quite useful in facilitating the development of these drugs.

Because similar priming by capped RNAs also apparently occurs in the infected cell, we can explain the α-amanitin-sensitive (RNA polymerase II) step required for viral RNA transcription in vivo (Lamb and Choppin, 1977; Spooner and Barry, 1977; Mark et al, 1979), i.e., the host RNA polymerase II is required for the synthesis of capped RNA primers (Bouloy et al, 1978; Plotch et al, 1979; Bouloy et al 1979; Robertson et al, 1980). Recently, we have been able to demonstrate directly that only those capped cellular RNAs made after infection and not those pre-existing before infection serve as primers. Using a new assay for pulse-labeled viral RNA transcripts in the infected cell, we have shown that the cap, which is donated by the host RNA primer, is synthesized only after and not before infection (Herz, Stavnezer and Krug, manuscript in preparation). An important question then is why only newly synthesized capped cellular RNAs serve as primers. One possibility is that pre-existing, but not newly synthesized, capped RNAs are tied up in ribonucleoprotein structures (including polyribosomes) and cannot be used by the viral transcriptase. If so, the viral transcriptase would be expected to function near the site of synthesis of capped cellular RNAs in the nucleus. Several lines of evidence have suggested that some steps in viral RNA transcription occur in the nucleus (Lamb and Lai, 1980; Mark et al, 1979; Krug et al, 1976; Barrett et al, 1979), and recent experiments using our new

assay for pulse-labeled viral RNA transcripts have established a nuclear site for viral RNA transcription (Herz, Stavnezer, Gurney and Krug, manuscript in preparation).

ACKNOWLEDGEMENTS

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REFERENCES

1. Baralle, F.E. (1977) Nature, 267, 279-281.
2. Barrett, J., Wolstenholme, A.J. and Mahy, B.W.J. (1979) Virology 98, 211-225.
3. Bouloy, M., Morgan, M.A., Shatkin, A.J. and Krug, R.M. (1979) J. Virol., 32, 895-904.
4. Bouloy, M., Plotch, S.J. and Krug, R.M. (1978) Proc. Natl. Acad. Sci. USA, 75, 4886-4890.
5. Bouloy, M., Plotch, S.J. and Krug, R.M. (1980) Proc. Natl. Acad. Sci. USA, 77, 3952-3956.
6. Caton, A.J. and Robertson, J.S. (1980) Nucleic Acids Res. 8, 2591-2603.
7. Compans, R.W., Content, J. and Duesberg, P.H. (1972) J. Virol. 10, 795-800.
8. Darnell, J.E., Jr. (1978) Science 202, 1257-1260.
9. Dasgupta, R., Harada, F. and Kaesberg, P. (1976) J. Virol., 18, 260-267.
10. Dhar, R., Chanock, R.M. and Lai, C.J. (1980) Cell, 21, 495-500.
11. Furiuchi, Y., LaFiandra, A. and Shatkin, A.J. (1977) Nature, 266, 235-239.
12. Hay, A.J., Lomniczi, B., Bellamy, A.R. and Skehel, J.J. (1977) Virology, 83, 337-355.
13. Inglis, S.S., Carroll, A.R., Lamb, R.A. and Mahy, B.W.J. (1976) Virology 74, 489-503.
14. Koper-Zwarthoff, E.C., Lockard, R.E., Alzner-DeWeerd, B., RajBhandary, U.L. and Bol, J.F. (1977) Proc. Natl. Acad. Sci. USA, 74, 5504-5508.
15. Krug, R.M., Broni, B.A. and Bouloy, M. (1979) Cell, 18, 329-334.
16. Krug, R.M., Broni, B.A., LaFiandra, A.J., Morgan, M.A. and Shatkin, A.J. (1980).

17. Krug, R.M., Morgan, M.A. and Shatkin, A.J. (1976) J. Virol., 20, 45-53.
18. Krug, R.M., Plotch, S.J., Ulmanen, I., Herz, C. and Bouloy, M. (1981). In "Negative Strand Viruses" (R. Compans and D.H.L. Bishop, eds.) Elsevier/North Holland, in press.
19. Lamb, R.A. and Choppin, P.W. (1977) J. Virol., 23, 816-819.
20. Lamb, R.A. and Lai, C.J. (1980) Cell, 21, 475-485.
21. Lockard, R.E. and RajBhandary, U.L. (1976) Cell, 9, 747-760.
22. Mark, G.E., Taylor, J.M., Broni, B.A. and Krug, R.M. (1979) J. Virol., 29, 744-752.
23. Nevins, J.R. and Winkler, J.J. (1980) Proc. Natl. Acad. Sci. USA, 77, 1893-1897.
24. Plotch, S.J., Bouloy, M. and Krug, R.M. (1979) Proc. Natl. Acad. Sci. USA, 76, 1618-1622.
25. Plotch, S.J., Bouloy, M., Ulmanen, I. and Krug, R.M. (1981) Cell, 23, 847-858.
26. Robertson, H.D., Dickson, E., Plotch, S.J. and Krug, R.M. (1980) Nucleic Acids Res., 8, 925-942.
27. Robertson, J.S. (1979) Nucleic Acids Res., 6, 3745-3757.
28. Rochovansky, D. (1976) Virology, 73, 327-338.
29. Shatkin, A.J. (1976) Cell, 9, 645-653.
30. Shimotohno, K., Kodama, Y., Hashimoto, J. and Miura, K. (1977) Proc. Natl. Acad. Sci. USA, 74, 2734-2738.
31. Skehel, J.J. and Hay, A.J. (1978) Nucleic Acids Res., 4, 1207-1219.
32. Smith, J.C., Raper, R.H., Bell, L.D., Stebbing, N. and McGeoch, D. (1980) Virology, 103, 245-249.
33. Spooner, L.L.R. and Barry, R.D. (1977) Nature, 268, 650-652.

INFLUENZA VIRUS-SPECIFIC PROTEINS REGULATING GENOME TRANSCRIPTION [1]

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I. INTRODUCTION

The influenza virus genome consists of eight negative single-stranded RNA segments which range in size from 890 to approximately 2400 nucleotides (1). Transcription and replication of these genome RNA segments is strictly regulated in permissive host cells, as for example during infection of chick embryo fibroblast cells by the avian influenza A virus, fowl plague Rostock (2-4). In such infections, three distinct replication stages have been delineated by studying protein synthesis following release from a cycloheximide block: -

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1) Primary Transcription. This stage can be observed in the absence of virus-specific protein synthesis, and results in the formation of mRNAs specific for each of the eight RNA segments at approximately equivalent rates of synthesis. Subgenomic mRNAs encoded in segments 7 and 8 apparently require virus-specific protein syntehsis for their production and are not produced during primary transcription. All complementary RNA molecules (mRNAs) synthesised in the absence of virus-specific protein synthesis are polyadenylated incomplete copies of the genome RNA (6); their transcription stops at a U-rich polyadenylation signal located approximately 20 nucleotides from the 5' terminus of each genome RNA segment (7).

2) Early Secondary Transcription. During this stage there is selective amplification of transcription of segments 5 and 8, resulting in an increased rate of synthesis of the nucleoprotein (NP) and non-structural protein 1 (NS_1). These two proteins migrate rapidly into the nucleus after synthesis. Synthesis of non-polyadenylated full-length complementary RNA (A(-)cRNA) begins, and provides a template for the amplification of negative strand virion RNA molecules.

3) Late secondary transcription. The final stage in virus replication is characterised by an increased rate of transcription of segments 4, 6 and 7, encoding the haemagglutinin (HA), neuraminidase (NA) and matrix (M) proteins respectively. At this time, synthesis of proteins directed by the subgenomic spliced mRNAs encoded in segments 7 and 8 can be detected (8-10).

From the foregoing it is clear that certain virus-specified proteins are essential for the regulation of genome transcription as it occurs in normal infection. We have approached the problem of their identification by studies of fowl plague virus replication in chick embryo fibroblast cells, since we have available a large number of conditional lethal (ts) mutants, many of which are defective in virus-specific RNA synthesis at the restrictive temperature.

II. MATERIALS AND METHODS

The Rostock strain of avian influenza A (fowl plague) virus (A/Fowl plague virus /Rostock/34, H7NI) was used to infect primary chick embryo fibroblast or MDCK cell cultures. The ts mutants referred to in this study were originally isolated by J.W. Almond in this laboratory (11), and were plaque purified before use. The leak yield of the mutants at the restrictive temperature (40.5 °C) in all cases was less than 10^{-3} of the wild-type yield. Other methods used in this study have been detailed elsewhere (2,4,5,12), as will be indicated in the legends to the figures and tables.

III. RESULTS

A) Evidence that Transcription Occurs in the Cell Nucleus

We have previously presented evidence that the concentration of influenza virus-specific A(+) cRNA sequences is higher in the nucleus than in the cytoplasm at early times after infection (13). Further support for the view that the nucleus is the site of early influenza virus-specific RNA synthesis was provided by experiments with toyocamycin, an adenosine analogue. This compound has been found to prevent nuclear processing of adenovirus-specific RNA (14) as well as ribosomal RNA (15). We found that a concentration of 40 µg/ml toyocamycin in the culture medium caused approximately 1000-fold reduction in influenza virus yield during growth in MDCK cells. However, even at concentrations up to 400 µg/ml, no effect was observed on RNA transcription by purified influenza virus in vitro.

Table 1

Effect of Toyocamycin (40 ug/ml) on Polyadenylated Virus-Specific RNA Synthesis in Influenza Virus-Infected MDCK Cells [a]

		Virus-Specific RNA (pg A (+) cRNA per µg cell RNA)	
	Drug Treatment	Cytoplasm	Nucleus
Expt.1	None	110	108
	Toyocamycin	9	57
Expt.2	None	286	288
	Toyocamycin	24	271

[a] RNA was analysed from MDCK cells infected with influenza virus for 6 hours in the presence or absence of toyocamycin (40 ug/ml). The concentration of A (+) cRNA was measured using ^{125}I vRNA as described by Barrett et al. (4).

Analysis of virus-specific RNA produced in influenza virus-infected MDCK cells in the presence of 40 µg/ml toyocamycin showed that no A(-) cRNA or vRNA was detectable. However, up to 50 % of the normal amount of A(+) cRNA was synthesised in the presence of the drug, and was almost entirely confined to the cell nuclear fraction (Table 1). These data support previous evidence that influenza virus mRNA is synthesised in the nucleus (4,13,16,17).

B) Virus-specific proteins in the cell nucleus

Since early influenza mRNA synthesis occurs in the nucleus, those virus-specific proteins responsible for regulation of the transcriptional process should also be nucleus-associated. It has long been known that two virus-specific proteins, the NP and

NS proteins, migrate into the nucleus after synthesis in the cytoplasm (18,19). We wished to determine whether other virus-specific proteins such as the three P proteins, which were not recognised at the time of these early studies, also became associated with the cell nucleus. To do this we developed a fractionation method for MDCK cells using three detergents, which rapidly yielded nuclei judged intact and relatively free of cytoplasmic contamination by electron microscopic criteria (20). The rapidity of the technique enabled us to label virus-specific proteins for short periods (1 minute) with ^{35}S-methionine before chasing in the presence of a large excess of unlabelled methionine. These results showed that four virus-specific proteins were tightly associated with the nuclear fraction. Using two strains of fowl plague virus in which functionally equivalent P proteins (by recombinant analysis) migrate in a different order, the correspondent proteins were found to be nucleus-associated (Table 2). No evidence was found for association of the second non-structural protein with cell nuclei.

Table 2

Proteins which Specifically Migrate into the Cell Nucleus After Synthesis

Influenza Virus Strain	Proteins in Nucleus
Fowl Plague Dobson (H7N7)	P_1, P_3, NP, NS_1
Fowl Plague Rostock (H7N1)	P_1, P_2, NP, NS_1

Data summarized from Briedis et al. (20); proteins P_3 of the Dobson Strain and P_2 of the Rostock Strain are known to be functionally equivalent from recombinant analysis (27).

C) Primary Transcription by P Protein Mutants

The above results suggest that the P_1 and P_2 proteins, together with NP and NS_1, are likely to be involved in the regulation of mRNA transcription. We have previously shown that no overall reduction in A(+) cRNA synthesis occurs in cells infected at the non-permissive temperature with ts mutants mN3 and ts 47 (12). However, the site of the ts mutation on segment 8 is not known, and could affect the function of either the NS_1 or the NS_2 protein, or both, since the reading frames overlap (8,9).

A number of P protein mutants were screened for their ability to induce A(+) cRNA synthesis at the non-permissive temperature (40.5° C) under primary transcription conditions using cycloheximide to block virus-specific protein synthesis (Table 3). Although one mutant (ts45) defective in the P_3 protein, synthesised similar amounts of A(+) cRNA at either 34°C or 40.5 C, one P_1 and six P_2 mutants were all defective and synthesised 53 % or less A(+) cRNA at 40.5 °C compared to 34 °C. Interestingly, P_3 is the protein which remains in the cytoplasm after synthesis, and although firm conclusions cannot be drawn from a single mutant, this result is consistent with the hypothesis that only P_1 and P_2 of the Rostock strain are directly involved in primary transcription. (Table 3).

Other studies have shown that the pattern of protein synthesis induced by ts 45 at 40.5 C is similar to that seen immediately following release of normal wt virus-infected cells from a cycloheximide block (21). For example, protein NS_2 is not synthesised at all. This suggests that mutant ts 45 can complete the primary transcription stage at 40.5 °C but is blocked at the stage of secondary transcription. The fact that all six P_2 mutants so far examined are variously defective in primary transcription at 40.5 °C suggests that a functional P_2 protein may be essential for this process. This was confirmed when RNA transcriptase activity by purified mutant virions was analysed *in vitro*.

Table 3

Primary Transcription by Influenza Virus (Fowl Plague, Rostock) ts Mutants

Virus	Defective Protein	Incubation Temperature °C	ng Virus-Specific RNA per µg Cell RNA [a]
wt	-	34	0,86
		40,5	0,89 (104 %) [b]
ts 15	(P_1)	34	0,24
		40,5	0,11 (46 %)
ts 3	(P_2)	34	0,42
		40,5	0,15 (36 %)
ts 5	(P_2)	34	1,00
		40,5	0,53 (53 %)
ts 17	(P_2)	34	0,98
		40,5	0,28 (29 %)
ts 21	(P_2)	34	0,46
		40,5	0,20 (43 %)
ts 44	(P_2)	34	0,33
		40,5	0,15 (45 %)
ts nM5	(P_2)	34	0,23
		40,5	0,08 (34 %)
ts 45	(P_3)	34	0,46
		40,5	0,50 (108 %)

[a] Confluent CEF cell monolayer cultures were infected with approximately 20 p.f.u. per cell at either 34°C or 40,5 °C. Both cells and virus were separately incubated by immersion in a water bath at the appropriate temperature for 1 hour prior to infection. Cycloheximide (100 µg/ml) was present throughout. At 5 hours post-infection total cellular RNA was extracted, and the amount of virus-specific cRNA determined by hybridisation to ^{125}I-labelled virion RNA (vRNA) as previously described (4).

[b] percentage of the 34°C value.

Table 4

Globin mRNA priming of transcription by fowl plague virions *in vitro* at 40,5°C [a]

Incubation Time (min.)	- globin mRNA	+ globin mRNA [b]
0	604	850
15	640	10,156
30	657	10,436

[a] The activity of wild type virion transcriptase was assayed as described (12) except that ApG was omitted from the reaction mixture. Results are expressed as ^{3}H-GMP counts/min incorporated into acid-insoluble material per 10 ul sample.

[b] 15 µg globin mRNA was added to 150 µl reaction mixture.

D. Initiation of Transcription *in vitro*

There is now abundant evidence that the initiation of influenza virus-specific RNA synthesis in infected cells is primed by capped eukaryotic mRNA molecules (9, 22-24). Either capped mRNA molecules, or certain dinucleotides particulary ApG complementary to the 3' terminus of virion RNAs, stimulate influenza virion transcriptase activitv in an *in vitro* assay (25,26). We have screened representative *ts* mutants of all six available groups (11) for their ability to respond to ApG or capped globin mRNA by increased *in vitro* transcriptase activity (21). The only *ts* mutants found to obviously negative for priming *in vitro* by ApG or globin mRNA were in group III, with *ts* lesions in RNA segment 1 encoding protein P2.

At 40.5°C, the activity of the influenza virion transcriptase is minimal under standard assay conditions (12), but , as shown in Table 4, wild-type virus responds dramatically to addition of globin mRNA to the *in vitro* reaction.

Table 5

In vitro transcriptase activity of fowl plague Rostock ts mutants defective in protein P_2 [a]

	31 °C			40,5 °C	
Virus	Unprimed	ApG	mRNA	ApG	mRNA
wt	450	2300	1350	390	80
ts 9	600	1950	1000	320	<10
ts 44	300	1200	500	160	12
ts 3	150	1000	280	80	<10
ts 5	130	750	200	60	<10
ts 17	80	950	450	50	<10
ts 21	50	900	300	20	<10

[a] RNA polymerase activity was assayed at 31 °C or 40,5 °C as described (12) with addition of either 0,3 mM ApG or 100 µg/ml rabbit globin mRNA as indicated. Results are expressed as p moles ^{3}H-ump incorporated per mg virus protein during 60 min incubation.

Using this assay system, it was found that none of the six P_2 mutants studied could be primed by addition of globin mRNA in vitro at 40.5° C. although all responded at 31°C. We also assayed the transcriptase activity of these mutants at both temperatures in the presence of 0.3 mM ApG. Examples of these assays are given in figure 1 which shows ts 9 priming by ApG and not by globin mRNA at 40.5 °C, and ts 21 which cannot be primed at the restrictive temperature by either molecule.

IV. DISCUSSION

Several groups of investigators have attempted to define, using ts mutants, which influenza virus-specific proteins are involved in RNA transcription (reviewed in ref. 28). All studies have indicated a requirement for the P proteins and the nucleoprotein in transcription, but the precise function of each

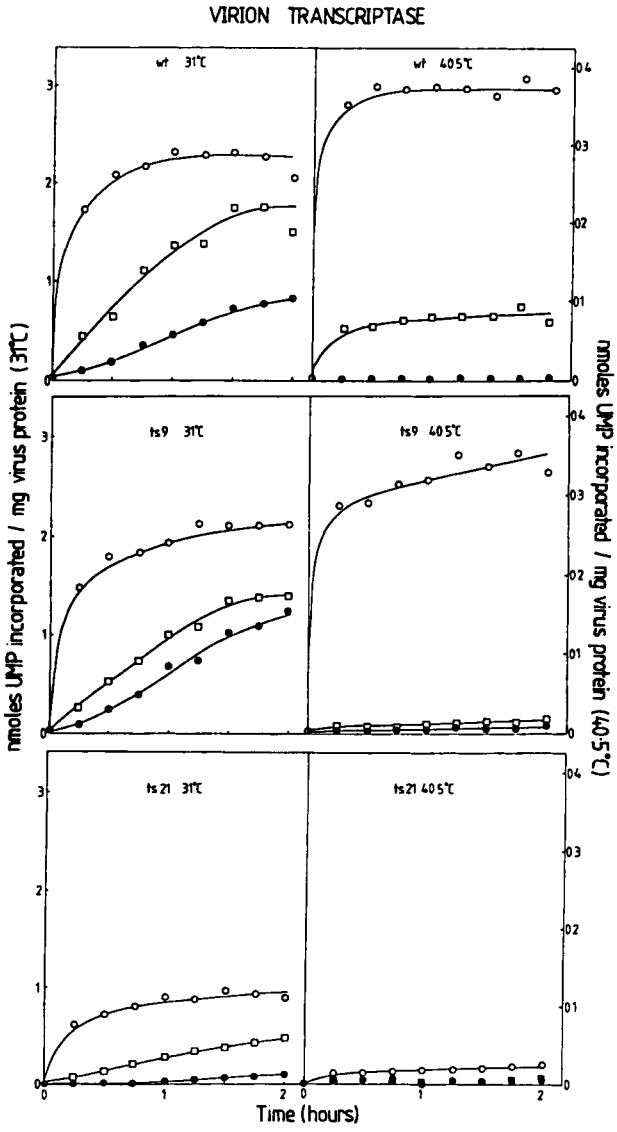

FIGURE 1. RNA transcription in vitro by wt and representative P2 mutants of fowl plague virus Rostock. The assay was as described (12), with either no primer ●——●, 0,3 mM ApG o——o or 100 µg/ml rabbit globin mRNA (□—□).

protein remains unclear. An extensive study of FPV Rostock ts mutants revealed that out of eighteen mutants tested four, all segment 1 mutants, were negative for ApG primed transcriptase activity at 40.5 °C, indicating that for this strain of FPV, a functional P2 protein (coded for by RNA segment 1) was required for virion transcriptase activity. When mutants were tested for mRNA stimulated transcriptase activity at 40.5 °C, two other mutants, ts 9 and ts 44, also appeared to be negative, suggesting that the P2 protein of this influenza virus strain is directly involved in the mRNA priming event, as well as in the transcription reaction itself. The mRNA priming mechanism apparently involves recognition of the 5' methyl cap structure and a specific endonucleolytic cleavage of primer molecules (29). The exact role of the P2 protein in the priming mechanism is as yet unclear, but is currently under investigation.

Comparison of data obtained with different influenza virus strains is complicated by the differences in electrophoretic mobility of equivalent genes and proteins (1). However, as it has been demonstrated that RNA segment 1 of fowl plague virus Rostock is functionally equivalent to segment 2 of WSN strain of influenza virus (28) there is a clear correlation between the results presented here and those obtained in previous studies with influenza WSN (30), influenza A/Ann Arbor/6/60 (31), and fowl plague virus Weybridge (32). In all cases, a protein encoded in the genome RNA segment functionally equivalent to influenza WSN RNA segment 2 is essential for the function of the virion transcriptase.

The fact that ts mutants from one recombination group can display more than one phenotype (28, 33) demonstrates the importance of analyzing a series of ts mutants from each recombination group before assigning minimum functions to the defective protein of that group. Thus this type of analysis does not rule out the possible role of other virus-specific proteins in the transcription process, or other roles for the P2 protein. Indeed we now know that purified influenza virions are capable of performing several activities, including cap recognition and endonucleolytic cleavage of primer RNA molecules (29) transcription of template RNA and

poly A synthesis (1,6), and phosphorylation of virion proteins (34,35). The exact role of each of the P proteins and NP protein in these processes should become clear from further genetic and phenotypic analysis of ts mutants.

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REFERENCES

1. Barry, R.D. and Mahy, B.W.J. (1979). Brit. Medical Bull. 35, 39.
2. Inglis, S.C., Carroll, A.R., Lamb, R.A. and Mahy, B.W.J. (1976). Virology 74, 489.
3. Hay, A.J., Lomniczi, B., Bellamy, A.R. and Skehel, J.J. (1977). Virology 83, 337.
4. Barrett, T., Wolstenholme, A.J. and Mahy, B.W.J. (1979). Virology 98, 211.
5. Inglis, S.C. and Mahy, B.W.J. (1979). Virology 95, 154.
6. Hay, A.J. and Skehel, J.J. (1979). Brit. Medical Bull. 35, 47.
7. Robertson, J.S., Schubert, M., and Lazzarini, R.A. (1981). J. Virol. 38, in press.
8. Inglis, S.C., Gething, M.J. and Brown, C.M. (1980). Nucleic Acids Res. 8, 3575.
9. Lamb, R.A. and Lai, C.J. (1980). Cell 21, 475.
10. Lamb, R.A., Briedis, D.J., Choppin, P.W., and Lai, C.J. (1981)., this volume.
11. Almond, J.W., McGeoch, D., and Barry, R.D. (1979). Virology 92, 416.
12. Wolstenholme, A.J., Barrett, T., Nichol, S.T. and Mahy, B.W.J. (1980). J. Virol. 35,1.
13. Barrett, T., Brownson, J.M., Wolstenholme, A.J., and Mahy, B.W.J. (1978)., in "Negative Strand Viruses and the Host Cell" (B.W.J. Mahy and R.D. Barry, eds). p 325. Academic Press, London.
14. Swart, C. and Hodge, L.D. (1978). Virology 84, 374.
15. Tavitian, A., Uretsky, S.C. and Acs, G. (1968). Biochim. Biophys. Acta 175, 33.
16. Krug, R.M., Morgan, M.A. and Shatkin, A.J. (1976). J. Virol. 20, 45.

17. Mark, G.E., Taylor, J.M., Broni, B. and Krug, R.M. (1979). J. Virol. 29, 744.
18. Taylor, J.M., Hampson, A.W. and White, D.O. (1969). Virology 39, 419.
19. Lazarowitz, S.G., Compans, R.W. and Choppin, P.W. (1971). Virology 46, 830.
20. Briedis, D.J., Conti, G., Munn, E.A. and Mahy, B.W.J. (1981). Virology in press.
21. Mahy, B.W.J., Barrett, T., Nichol, S.T., Penn, C.R. and Wolstenholme, A.J. (1981). in "Replication of Negative Strand Viruses" (D.H.L. Bishop and R.W. Compans, eds.). Elsevier North Holland, New York in press.
22. Krug, R.M., Broni, B.A. and Bouloy, M. (1979). Cell 18, 329.
23. Caton, A.J. and Robertson, J.S. (1980). Nucleic Acids Res. 8, 2591.
24. Dhar, R., Chanock, R.M. and Lai, C.J. (1980). Cell 21, 495.
25. McGeoch, D.J. and Kitron, N. (1974)., J. Virol. 15, 686.
26. Plotch, S.J., Bouloy, M. and Krug, R.M. (1979). Proc. Natl. Acad. Sci. USA 76, 1618.
27. Almond, J.W. and Barry, R.D. (1979). Virology 92, 407.
28. Scholtissek, C., Advances in Genetics 20, 1.
29. Plotch, S.J., Bouloy, M., Ulmanen, I., and Krug, R.M. (1981). Cell, in press.
30. Mowshowitz, S.L. (1978). Virology 91, 493.
31. Kendal, A.P., Cox, N.J., Galphin, J.C., and Maassab, H.F. (1979). J. gen. Virol. 44, 443.
32. Ghendon, Y.Z., Markushin, S.G., Blagovezhenskaya, O.V., and Genkina, D.B. (1975). Virology 66, 454.
33. Szilagyi, J.F. and Pringle, C.R. (1979). J. Virol. 30, 692.
34. Kamata, T. and Watanabe, Y. (1977). Nature 267, 460.
35. Nichol, S.T., Ph. D. Thesis, University of Cambridge

GENOME DIVERSITY AMONG INFLUENZA A, B AND C VIRUSES AND GENETIC STRUCTURE OF RNA 7 AND RNA 8 OF INFLUENZA A VIRUSES

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ABSTRACT. cDNA-RNA hybridization analysis of the RNAs of different influenza viruses shows that the genomes of type A strains vary more than those of type B strains and that the genomes of type B strains exhibit more variation than those of type C strains.

Comparison of the nucleotide sequences of RNA 8 of four different influenza A viruses reveals that the gene length of 890 nucleotides, the arrangement of the overlapping NS1 and NS2 genes and the putative RNA splicing signals are conserved. The NS RNAs of A/PR/8/34, A/FPV/Rostock/34 and A/Udorn/72 viruses contain open reading frames in their (-) RNAs potentially coding for polypeptides of 167, 167 and 216 amino acids respectively. The A/duck/Alberta/60/76 virus NS segment, which shows extensive nucleotide differences from the NS genes of the three other strains, lacks such an agnogene in its negative strand sequence.

Isoleucine labeling (but not methionine labeling) of influenza A/WSN/33 virus-infected cells reveals a virus-specific polypeptide of apparent MW of 15.7 K. The migration pattern of this polypeptide is changed when cells are infected with recombinants of A/WSN/33 virus in which the M genes are derived from different strains. These findings support the idea that RNA 7 of influenza A viruses codes for the virion associated M polypeptide and a smaller isoleucine-rich polypeptide.

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INTRODUCTION

Although there is little or no serologic cross-reactivity between members of different influenza virus types, all type A strains share serologically crossreacting internal proteins, and all type B strains possess crossreacting internal proteins, which differ from those of type C influenza viruses (for review, 1). In addition, it was found that influenza virus strains belonging to one type have genomes of similar size, as demonstrated by polyacrylamide gel electrophoresis of their RNAs in the presence or absence of glyoxal (2,3). Also, it is well recognized that members of all three influenza virus types undergo antigenic and genetic variation during passage in nature. Such changes have been identified using serologic techniques, RNA-RNA hybridization analyses, oligonucleotide fingerprinting and protein and nucleic acid sequencing. However, little is known about the extent of diversity among the genomes of influenza B and C virus strains in comparison with that found for different influenza A viruses. Our results based on cDNA-RNA hybridization analyses of different influenza viruses suggest that the genomes of type A strains are the most variable and the genomes of type C strains are the most conserved.

In another study we compared the genetic structure of the NS genes of different influenza A viruses by sequence analysis. Our results confirmed the presence of overlapping NS1 and NS2 genes on RNA 8 of different strains. Furthermore, examination of the virion (-) strand of different influenza A virus NS genes revealed open reading frames with coding potentials of 167 amino acids in the A/PR/8/34 and A/FPV/Rostock/34 virus NS genes and of 216 amino acids in the A/Udorn/72 NS gene.

Finally, we wish to report preliminary results concerning the expression of a second M polypeptide in influenza virus infected cells. These findings confirm the suggestion made earlier by Winter and Fields (4) and Allen et al. (5) that RNA 7 of influenza A viruses may code for two distinct polypeptides.

RESULTS AND DISCUSSION

Comparison of the genomes of influenza A, B and C viruses

Table 1 shows estimates of base sequence homology of representative influenza A, B and C viruses using cDNA hybridization probes prepared from viral RNAs with DNase-digested calf thymus DNA as primer and reverse transcriptase (6). Under the hybridization and assay conditions used, no appreciable homology between the genomes of the three influenza virus types was detected. By RNA-RNA hybridization Scholtissek and coworkers reported that the genes of fowl plague virus and influenza B viruses may share up to 50% homology (7,8). We feel that our assay conditions are more stringent and that consequently homology values based on cDNA-RNA hybridization and S1 nuclease treatment are lower than those determined by RNA-RNA hybridization. It is thus difficult to directly compare the results obtained by these two hybridization techniques.

Sequence homology (as determined by S1 nuclease resistance of the cDNA-RNA hybrids) ranged from 15-100% among different influenza A viruses, 79-100% among influenza B viruses and 90-100% among influenza C viruses (Table 2). It should be noted that virus isolates covering a similar time span, i.e. about 30 years, were used in these analyses. One possible explanation for less variation among influenza B and C viruses is that these viruses are apparently restricted in serial transmission to humans, and no "true" animal isolates of these viruses have been made. Hence these viruses cannot reassort their genes with those of animal strains as occurs with the influenza A viruses. Alternatively it is possible that influenza B and C viruses have had fewer generations in nature than influenza A viruses, and therefore the chances of a mutation occurring in their genomes have been decreased. A third possibility is that the RNA polymerase of influenza B and C viruses is less error prone than the type A virus polymerase, resulting in more conservation of influenza B and C virus gene sequences.

We appreciate, however, that the probes used in our study may not be representative of all regions of all the RNA segments, because the

TABLE 1

HYBRIDIZATION OF INFLUENZA A, B AND C VIRUS RNAs TO ^{32}P-LABELLED cDNAs[a]

	S1 nuclease resistance of hybrids with cDNA prepared to RNA from:		
Protecting RNA	A/PR/8/34	B/HK/8/68	C/NJ/76
A/PR/8/34	100	3	2
B/HK/8/68	2	100	1
C/NJ/76	2	3	100

[a] ^{32}P-labeled cDNAs were synthesized in 50 µl reactions containing 50 mM Tris HCl, pH 8.3, 10 mM $MgCl_2$, 10 mM DTT, 1 mM each dATP, dGTP and TTP, 0.1 mM dCTP, 100 µCi α-^{32}P-dCTP (NEN, Sp. act. 600 Ci/mmol), 100 µg/ml actinomycin D, 5 µg vRNA and 50 µg calf thymus DNA primers. The reaction mixture was incubated at 37°C for 2 hours with 20 units of reverse transcriptase. The reaction was terminated by adding 150 µl of 0.75% SDS and 300 µl 0.5 M NaOH, and the RNA hydrolyzed by overnight incubation at 37°C. The cDNA was purified by Sephadex G50 chromatography and concentrated by ethanol precipitation. Hybridizations were performed in 10 µl volumes, containing 0.3 M NaCl, 0.01 M Tris HCl pH 7.0, 1 mM EDTA and 0.5% SDS, 100 ng vRNA and about 5000 cpm of cDNA. The solutions were heated at 100°C for 3 minutes and then incubated at 68°C for 1 hour. 400 µl of S1 nuclease buffer (30 mM sodium acetate, pH 4.6, 50 mM NaCl, 2 mM $ZnSO_4$, 5% glycerol) were added. Two 200 µl aliquots were taken, and 20 units of S1 nuclease (Sigma) were added to one aliquot. Both samples were incubated at 45°C for 30 minutes, when 1 ml of 10% TCA and 10 µg yeast RNA were added. After 30 minutes incubation on ice, precipitated nucleic acid was collected by filtration and dried filters were counted in a scintillation counter. Hybridizations were performed in duplicate and the values are the mean of two experiments.

TABLE 2

HYBRIDIZATION OF INFLUENZA A VIRUS RNAs TO A/PR/8/34 VIRUS cDNA, INFLUENZA B VIRUS RNAs TO B/HK/8/73 VIRUS cDNA AND INFLUENZA C VIRUS RNAs TO C/TAYLOR/47 VIRUS cDNA.[a]

Protecting RNA	% S1 Nuclease Resistance
A/PR/8/34 (H1N1)	100
A/WSN/33 (H1N1)	90
A/Singapore/1/57 (H2N2)	52
A/Aichi/2/68 (H3N2)	46
A/duck/France/MA42/76 (H6N2)	26
A/equine/Prague/1/56 (H7N7)	15
B/HK/8/73	100
B/HK/5/72	103
B/Victoria/70	102
B/Russia/69	94
B/Singapore/64	91
B/Taiwan/62	85
B/Gt Lakes/54	93
B/Lee/40	79
C/Taylor/47	100
C/Gt Lakes/54	99
C/Johannesburg/66	91
C/New Jersey/76	91

[a]Hybridizations with calf thymus DNA primed cDNA probes were performed as described in Table 1.

templates are not present in equimolar amounts or because of some unfavorable secondary structure of the template which affects reverse transcription. Also, by looking at the hybridization of a "total" probe rather than individual gene probes, the extreme variation of an individual RNA segment could be masked. Thirdly, although we have compared viruses isolated over a similar wide time span, it is possible that other isolates of influenza B and C viruses may exhibit more variation than those shown in Table 2.

Structure of RNA segment 8

In a second study we determined the complete nucleotide sequences of cloned NS genes of A/PR/8/34 and A/duck/Alberta/60/76 viruses (9,10) and compared them with the previously sequenced NS genes of A/FPV/Rostock/34 (11) and A/Udorn/72 (12) viruses. The results summarized in Table 3 reveal that the differences among the nucleotide sequences of the A/PR/8/34, A/FPV/Rostock/34 and A/Udorn/72 viruses range from 8-11%. In contrast, the NS gene of the A/duck/Alberta/60/76 virus differs from those of the other three viruses by approximately 27%. Despite the large number of nucleotide changes detected, the conserved features of the four NS genes include a gene length of 890 nucleotides, the coding capacity of overlapping NS1 and NS2 genes, the putative RNA splicing signals and the presence of a potential transcription termination and polyadenylation signal (9,10).

As we reported earlier (9), the A/PR/8/34, A/FPV/Rostock/34 and A/Udorn/72 viruses have the coding potential for a third NS polypeptide in the virion RNA. Figure 1 shows that there is one open reading frame of 501 nucleotides on the (-) RNA of the NS gene of A/PR/8/34 virus, and the two open reading frames on the (+) RNA which give rise to the NS1 and NS2 polypeptides. The reading frames on the virion RNAs of the NS genes of A/PR/8/34, A/FPV/Rostock/34, A/Udorn/72 and A/duck/Alberta/60/76 viruses are shown in Figure 2. It seems unlikely that open reading frames spanning 501 nucleotides in the NS genes of A/PR/8/34 and A/FPV/Rostock/34 viruses or 648 residues in that of the Udorn virus would occur by chance, since the probability of finding open reading frames with these lengths is 0.0003 and 0.00003 respectively (9). These probabilities are based on the random selection of the 64 codons, three of which are termination codons. On the same basis one would predict the occurrence of 8 and 10 termination codons in regions containing 167 and 216 triplets, respectively. This prediction is confirmed by the observation of 6-14 termination codons within the other reading frames that span the presumptive NS3 genes (Fig. 2). In addition, another group has independently cloned and

TABLE 3

DIFFERENCES IN NUCLEOTIDE SEQUENCES AMONG THE NS GENES OF FOUR INFLUENZA VIRUSES

NS Gene Derivation	Total (%)	NS1 (%)	NS2 (%)
A/PR/8/34-A/Udorn/72	78/890 (8.8)	63/690 (9.1)	29/363 (8.0)
A/PR/8/34-A/FPV/Rostock/34	71/890 (8.0)	61/690 (8.8)	24/363 (6.6)
A/Udorn/72-A/FPV/Rostock/34	96/890(10.8)	80/690(11.6)	31/363 (8.5)
A/PR/8/34-A/duck/Alberta/60/76	243/890(27.3)	210/690(30.4)	69/363(19.0)
A/duck/Alberta/60/76-A/FPV/Rostock/34	236/890(26.5)	198/690(28.7)	65/363(17.9)
A/duck/Alberta/60/76-A/Udorn/72	245/890(27.5)	206/690(29.9)	72/363(19.8)

The lengths of the NS genes, NS1 coding regions and NS2 coding regions are 890, 690 and 363 nucleotides respectively. The differences reported for the NS1 coding regions were calculated only for the "common" 690 nucleotides. It should be noted that the Udorn virus NS1 is 21 nucleotides longer than those of the other viruses. The nucleotide sequences for the NS genes of A/PR/8/34, A/duck/Alberta/60/76, A/FPV/Rostock/34 and A/Udorn/72 viruses were reported by Baez et al. (9), Baez et al. (10), Porter et al. (11) and Lamb and Lai (12), respectively.

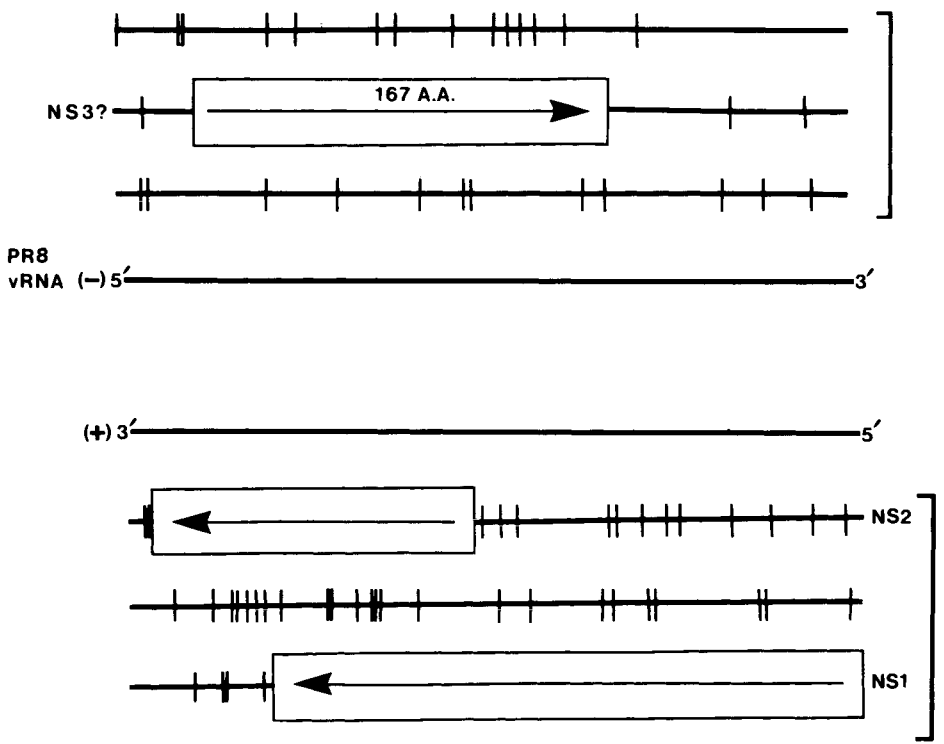

FIG. 1. Reading frames of the influenza A/PR/8/34 virus NS gene. The top panel shows the three reading frames of the (-) sense RNA (virion RNA). Vertical lines indicate stop codons. The open box delineates an open reading frame of 501 nucleotides starting with AUG at the 5' end. The bottom panel indicates reading frames for the (+) sense RNA, which lead to the translation of NS1 and NS2 polypeptides.

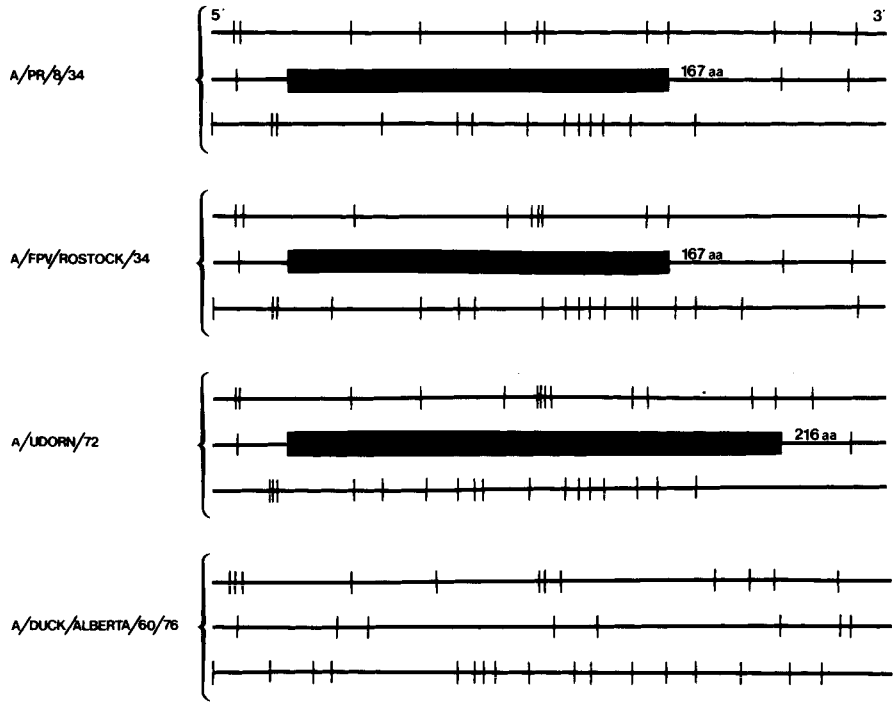

FIG. 2. Examination of open reading frames in the virion (-) RNA of four influenza virus NS genes. Vertical lines indicate the positions of termination codons and long dark rectangles represent open reading frames of 501 and 648 nucleotides, respectively.

sequenced the NS gene of an A/PR/8/34 virus with a different passage history (13). The DNA sequence reported by this group shows the same open reading frame in the (-) RNA sequence of the NS gene. An examination of the (-) RNA sequence of the A/duck/Alberta/60/76 virus NS gene did not reveal an open reading frame similar to those previously identified in the NS genes of the other three (four) strains. Instead we found four termination codons within the region of the reading frame expected to contain the agnogene sequence (Fig. 2). Unless a

specific splicing mechanism to circumvent the stop signals is invoked, it appears that the A/duck/Alberta/60/76 virus NS gene does not have the coding potential for a third polypeptide. At the present time we cannot determine whether the absence of the open reading frame in the A/duck/Alberta/60/76 virus NS gene is specific to the DNA clone analyzed or if its absence is specific for the viral isolate used in this study. If subsequent analyses of the A/PR/8/34, A/Udorn/72 and other influenza viruses establish the existence of an NS3 polypeptide, it would suggest that the NS3 polypeptide is not essential for the survival of the A/duck/Alberta/60/76 virus.

Identification of a second polypeptide coded by RNA 7

The third topic which we wish to discuss in this paper concerns the genetic structure of RNA 7 of influenza A viruses and the polypeptides which may be coded for by this RNA. Winter and Fields (4) and Allen et al. (5) found by sequencing the M gene of A/PR/8/34 virus that this RNA segment possesses overlapping open reading frames, which may code for two polypeptides. Examination of the deduced amino acid sequence of the second M polypeptide (M2) revealed that isoleucine is the most abundant amino acid in this molecule. Hence we proceeded to label influenza virus infected cells with ^{3}H-isoleucine and found that in A/WSN/33 and in A/HK/8/68 virus-infected cells an isoleucine-rich protein migrated with apparent molecular weights of 15.7 K and 15.5 K respectively (Fig. 3). In contrast, ^{35}S-methionine labeling of cells made it difficult to identify such a polypeptide (data not shown). Since this isoleucine-rich polypeptide migrated in slightly different positions in A/WSN/33 and in A/HK/8/68 virus-infected cells, it seemed likely to be of viral origin (Fig. 3).

Further examination using recombinant viruses confirmed that this polypeptide is virus specific and encoded by the M gene. MDCK cells were infected with two recombinant viruses, W-N and W-H, and labeled with ^{3}H-isoleucine at 6 hrs. p.i. The recombinant W-H derives all genes from A/WSN/33 virus with the exception of the M gene which originates from A/HK/8/68 virus (14) and the recombinant

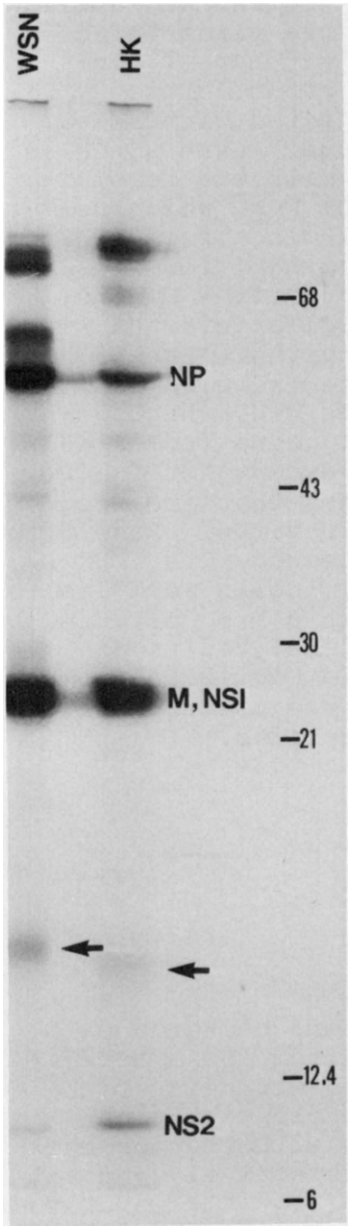

FIG. 3. Isoleucine labeling of influenza virus infected MDCK cells. Monolayers of MDCK cells were infected with A/WSN/33 or A/HK/8/68 viruses and at 13 hrs. p.i. ^{3}H-isoleucine (50 µCi/ml) was added for 60 min. Cells were lysed with SDS/2-mercaptoethanol, and proteins were separated on a 12% polyacrylamide gel containing 8 M urea. The positions of viral proteins and marker proteins are indicated to the right. Arrows point to the positions of the M2 polypeptides.

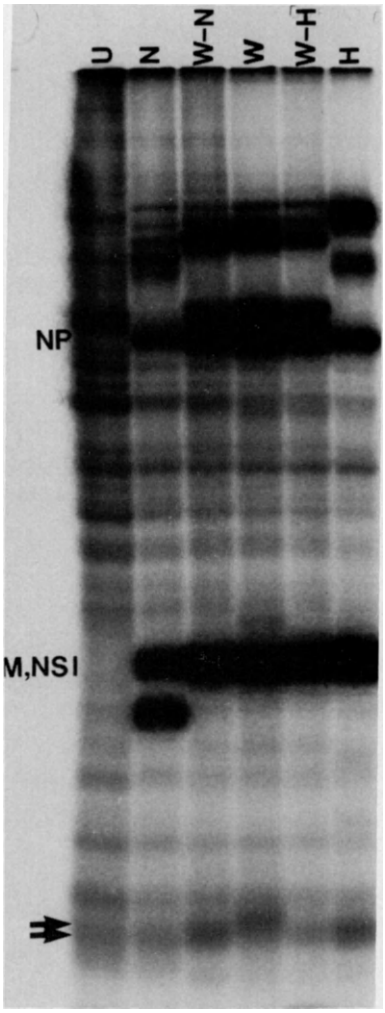

FIG. 4. Isoleucine labeling of influenza virus infected MDCK cells. Lane 1, uninfected cells. Lane 2, cells infected with A/Ned/84/68 virus. Lane 3, cells infected with the recombinant virus W-N, which derives all genes from A/WSN/33 virus except for the A/Ned/84/68 virus M gene. Lane 4, cells infected with A/WSN/33 virus. Lane 5, cells infected with recombinant virus W-H, which derives all genes from A/WSN/33 virus except for the Pl and M genes that are from A/ HK/8/68 virus. Lane 6, cells infected with A/HK/8/68 virus, Cells were labeled labeled at 6 hrs. p.i. and proteins analyzed as described in Fig. 3. Arrows indicate the position of the M2 polypeptides.

W-N also has an A/WSN/33 virus gene background with the Pl and M genes derived from the A/Ned/84/68 virus (14). In cells infected with either the W-N or the W-H recombinant the isoleucine-rich polypeptide appears to comigrate with that of the A/Ned/84/68 and A/HK/68 virus respectively and not with that of the A/WSN/33 virus (Fig. 4); as stated above, in neither recombinant is the M gene derived from the A/WSN/33 virus. This finding suggests that the isoleucine-rich polypeptide migrating between the M/NS1 polypeptides and the NS2 polypeptide is of viral origin and is coded for by RNA 7. It is likely that the M2 polypeptide is

encoded by the 5' portion of the M gene because the second reading frame in this region of the (+) RNA would direct the synthesis of an isoleucine rich-methionine poor protein. We are currently attempting to obtain additional proof to confirm the hypothesis of Winter and Fields (4) and Allen et al. (5) that RNA 7 of influenza A viruses codes for two distinct polypeptides which are expressed during the infectious cycle of the virus.

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REFERENCES

1. The Influenza Viruses and Influenza. (E.D. Kilbourne, ed.) 1975. Academic Press, New York.
2. Palese, P., Racaniello, V.R., Desselberger, U., Young, J.F. and Baez, M. (1980). Phil. Trans. R. Soc. Lond. B288, 299.
3. Desselberger, U. and Palese, P. (1978). Virology 88, 394.
4. Winter, G. and Fields, S. (1980). Nucl. Acids Res. 8, 1965.
5. Allen, H., McCauley, J., Waterfield, M. and Gething, M.J. (1980). Virology 107, 548.
6. Elliott, R.M. and Palese, P. (manuscript submitted).
7. Scholtissek C. and Rott, R. (1969). Virology 39, 400.
8. Scholtissek, C., Rohde, W. and Harms, E. (1977) J. Gen. Virol. 37, 243.
9. Baez, M., Taussig, R., Zazra, J.J., Young, J.F., Reisfeld, A. and Skalka, A.M. Nucl. Acids Res. 8, 5845.
10. Baez, M., Zazra, J.J., Elliott, R.M., Young, J.F. and Palese, P. (manuscript submitted).
11. Porter, A.G., Smith, J.C. and Emtage, J.S. (1980). P.N.A.S. 77, 5074.

12. Lamb, R.A. and Lai, C.-J. (1980). Cell 21, 475.
13. Winter, G., Fields, S., Gait, M.J. and Brownlee, G.G. (1981). Nucl. Acids Res. 9 237.
14. Ritchey, M.B. and Palese, P. (1977). J. Virol. 21, 1196.

MULTIPLE mRNAs AND CODING REGIONS DERIVED FROM INDIVIDUAL INFLUENZA A AND B VIRUS RNA SEGMENTS

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I. THE SEQUENCE AND ARRANGEMENT OF mRNAs CODING FOR THE TWO OVERLAPPING NONSTRUCTURAL PROTEINS OF INFLUENZA A VIRUS

The genome of influenza virus consists of eight single-stranded RNA segments (1-3), each of which is transcribed into mRNA that codes for a different polypeptide (4-6). These polypeptides include three associated with RNA transcriptase activity (P_1, P_2 and P_3), the hemagglutinin (HA), the nucleocapsid protein (NP), the neuraminidase (NA), the membrane protein (M) and a nonstructural protein NS_1 (7-10). In influenza virus-infected cells, we and others have observed a ninth polypeptide (Mr ∿11,000) now designated NS_2 (8, 11-13) which is synthesized late in infection (13).

We showed that NS_2 is a unique ninth influenza virus polypeptide on the basis of its peptide composition, the isolation of a separate mRNA for it and strain-specific differences in its migration on polyacrylamide gels (13-15). We postulated that one RNA segment must code for two polypeptides (13) because of the evidence for nine virus-coded polypeptides and the existence of only eight influenza virus RNA

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segments. This led to the finding that virus RNA segment 8 codes for both NS_1 and NS_2 as shown in studies with recombinant viruses in which NS_1 and NS_2 reassorted together and in experiments in which hybridization of virion RNA segment 8 to total virus mRNA specifically prevented the synthesis of both NS_1 and NS_2 in vitro (15, 16). As the size of RNA segment 8 was estimated to be 900 nucleotides (17) and as NS_1 (Mr ∿23,000) would need 615 nucleotides and NS_2 (Mr ∿11,000) 294 nucleotides to code for these two polypeptides, we suggested that different reading frames were being used to translate NS_1 and NS_2 (15). Using cloned DNA derived from RNA segment 8 (NS DNA) the mRNAs for NS_1 and NS_2 were mapped on RNA segment 8 (18) using the S_1 nuclease technique. These data indicated that the body of the NS_1 mRNA (∿850 nucleotides) maps from 0.05-0.95 units of the cloned NS DNA and the body of the NS_2 mRNA (∿340 nucleotides) maps from 0.59-0.95 units, suggesting that the two mRNAs are 3'-co-terminal and have the same poly(A) addition site. This location of the two mRNAs was confirmed by hybrid-arrested translation experiments using fragments of the cloned NS DNA to inhibit the synthesis in vitro of NS_1 and NS_2. In addition, we estimated from the size of the products of premature termination of translation of NS_1 in these experiments that termination of translation of NS_1 occurred at ∿0.76 map units. Therefore, we predicted that as NS_1 and NS_2 did not share common [^{35}S]-methionine or [^{3}H]-leucine containing tryptic or chymotryptic peptides, that NS_1 and NS_2 overlap by 50-60 amino acids that are translated from different reading frames (18).

Although the above experiments mapped the position of the bodies of the mRNAs for NS_1 and NS_2 it was possible that we had failed to detect the existence of small RNA segments, specifically any of less than 50 nucleotides. We therefore decided to examine the precise 5'-terminal nucleotides of the NS_2 mRNA and to demonstrate directly that translation of the NS_2 mRNA could occur in a reading frame different from that used for NS_1, by sequencing the cloned NS DNA and the NS_2 mRNA.

We obtained the complete sequence of both strands of the NS DNA (19) and showed that the NS DNA is a full length copy of influenza virus gene 8 as it contains both the 3'- and 5'-terminal sequences of the RNA segment (20). In addition, as the NS DNA clone was constructed from cDNA copies of both the virion RNA segment 8 and the NS_1 mRNA (21), the NS DNA contains 10-11 non-viral nucleotides which were derived from the 5'-end of the mRNA and are presumably derived from a cellular mRNA (22). Sequence analysis of independently cloned DNA

segments showed that these non-viral nucleotides are heterogeneous in length and sequence providing further evidence that cellular RNA sequences are used to prime influenza viral mRNA transcription in infected cells (23).

The sequence of the NS DNA (19) indicates that the colinear NS_1 mRNA consists of at least 864 nucleotides depending on the exact site of termination of transcription and polyadenylation. There is a 5'-non-coding region of 26 nucleotides before the first AUG followed by an open reading frame that could code for a protein of 237 amino acids, which is compatible with the size of the NS_1 polypeptide. The +1 reading frame is open from nucleotide 460 to 861, a region large enough to code for the NS_2 polypeptide, a conclusion supported by our previous data showing that the body of the NS_2 mRNA contained ∿340 nucleotides (18).

To investigate the nucleotide arrangement at the 5'-end of the NS_2 mRNA, this mRNA was sequenced by the primer extension method. A DNA primer from the body region of the NS_2 RNA was hybridized to purified NS_2 mRNA and the primer extended, with reverse transcriptase, using the mRNA as a template, and then the single-stranded DNA was sequenced. These data (19) showed that the 5'end of the NS_2 mRNA is heterogeneous in sequence for 10-20 nucleotides. Following this heterogeneous sequence there are ∿56 nucleotides that are complementary to the 3'-end of the virion RNA segment 8 and are the same nucleotides that are found at the 5'-end of the NS_1 mRNA. After this ∿56 nucleotide leader sequence, there is an interrupted region of 473 nucleotides, with the leader being covalently linked to the body of the NS_2 mRNA beginning at nucleotide 526-529. The exact nucleotide at which the 5'-terminal leader sequence is joined to the body of the NS_2 mRNA cannot be determined because of the repetition of CAGG at 54-57 and 526-629, but, as described below, it is likely to be nucleotides 56 and 529. This mRNA arrangement creates an open reading frame from the initiation codon of protein synthesis at nucleotide 27-29, and continues until the termination codon at 862-864. Thus 9 amino acids coded by nucleotides 27-56 would be shared by NS_1 and NS_2, and then translation of NS_2 would occur in the +1 reading frame after the interrupted region. The sequences indicate that NS_1 and NS_2 overlap by 70 amino acids that are translated from different reading frames. A schematic representation for the arrangement of the mRNAs for polypeptides NS_1 and NS_2 is shown in Fig. 1.

In eucaryotic mRNAs the nucleotides at both sides of

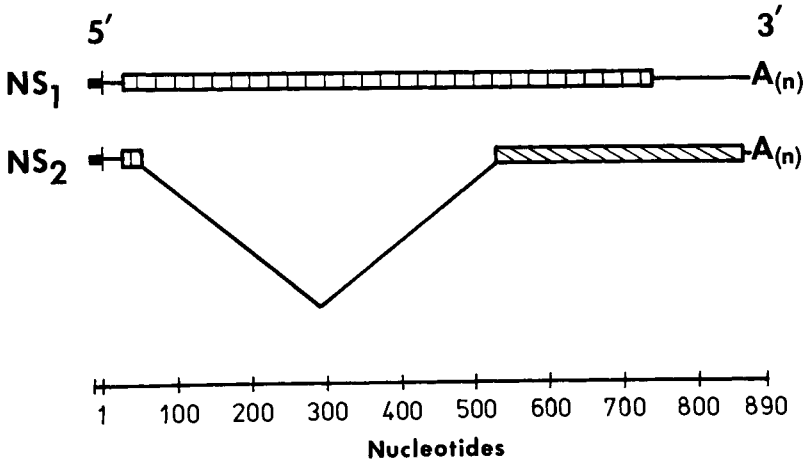

FIGURE 1. Schematic Representation for the Arrangement of the NS_1 and NS_2 mRNAs. The thin lines at the 5'- and 3'-termini of the NS_1 and NS_2 mRNAs represent noncoding regions. The thick shaded lines represent the coding regions of the two mRNAs. In the region 529-861 the NS_2 mRNA is translated in a reading frame different from that used for NS_1. The V-shaped thin line in the NS_2 mRNA represents the interrupted sequence in the NS_2 mRNA. The medium line and bar before nucleotide 1 at the 5'-termini represent the heterogeneous nucleotides derived from cellular mRNAs that are donated to the NS_1 and NS_2 mRNAs. [From Lamb and Lai (19).]

intervening sequences have been found to follow a distinct pattern. The 5'- (donor) site has the preferred form AG↓GTA (↓= cleavage), and the 3'- (acceptor) site has the preferred sequence PyPyNPyAG↓, with no AG in the preceding 10 nucleotides (24, 25, 26). These consensus sequences are found at the junctions of the intervening sequence in the NS_2 mRNA.

The synthesis of the NS_2 mRNA may occur by processing of its colinear transcript, NS_1 mRNA,by a splicing mechanism. If the NS_1 mRNA is a substrate for splicing, the event is tightly controlled, because most of the mRNA in the cytoplasm is NS_1 mRNA (18). In addition, we showed earlier that the synthesis of the NS_2 mRNA is dependent on early protein synthesis (13). For eucaryotic mRNAs, splicing is a nuclear event, and thus this processing may be an important nuclear event in influenza virus replication. It is possible that a pool of the NS_1 mRNA in the nucleus is modified in a subtle way, e.g., by methylation, which makes it a substrate for splicing. Alternative, but less likely, possibilities for the formation of the NS_2 mRNA have been discussed by us previously (19). These include transcriptional jumping exactly at the complement of the consensus sequence, or from formation of DI particles with internal deletions exactly at the complement of the consensus sequence. The nucleotide sequence of the NS DNA provides indirect evidence concerning the amino acid sequence of NS_1 and NS_2 in the absence of direct protein sequence information. NS_1 is predicted to have a mol. wt. of 26,815 and NS_2 a mol. wt. of 14,216. The amino acid composition of NS_1 obtained for the WSN strain (27), compares well with that predicted for the Udorn strain (19). In addition, the predicted tryptic or chymotryptic peptides containing methionine or leucine for NS_1 and NS_2 compare very well to those found previously (13, 15, 18).

II. INFLUENZA B VIRUS RNA SEGMENT 8 CODES FOR TWO NONSTRUCTURAL PROTEINS AND MORE THAN ONE mRNA MAY BE DERIVED FROM SEGMENT 7

Influenza A and B viruses differ in their epidemiology, immunological specificity of surface and internal proteins, and other biological characteristics (reviewed in ref. 28) including the observation that recombination between A and B viruses has not been demonstrated. Biochemically, influenza B viruses are similar to influenza A viruses in having 8 RNA segments (29) and which code for 8 viral polypeptides analogous to those of influenza A viruses (30-33). A ninth

polypeptide analogous to the NS_2 polypeptide of influenza A virus has been found in influenza B virus-infected cells (15, 32) but the virus-specificity of this polypeptide had not been conclusively shown nor the RNA segment that codes for it identified.

We have recently shown that the eighth genome RNA segment of influenza B virus codes for two nonstructural polypeptides (NS_1 and NS_2) (34). These polypeptides are translated from separate mRNA species which have been identified by in vitro translation after gradient separation. The location of the gene coding for the NS_2 polypeptide was determined by hybridization of its mRNA to individual virus genome RNA segments and the resulting specific inhibition of its synthesis in vitro after hybridization to genome segment 8 (Fig. 2).

[^{125}I]-labeled influenza B virus RNA segment 8 was hybridized to gradient mRNA fractions, treated with single-strand-specific ribonucleases, and analysed on gels (Fig. 3). Gradient fractions from which NS_1 could be translated gave rise to a predominant species of double-stranded RNA ∿1,100 base pairs in length, while fractions from which NS_2 was translated produced an additional species of double-stranded RNA ∿430 base pairs in length. These presumably correspond to the lengths, respectively, of the influenza B NS_1 and NS_2 mRNAs. If these estimates are correct, then the lengths of the influenza A and B virus NS_2 mRNAs would be remarkably similar. When [^{125}I]-labeled influenza B virus RNA segment 7 was used in a similar analysis, double-stranded RNA species were detected which would correspond not only to an mRNA of ∿1,250 nucleotides, but also to 2 shorter mRNAs of ∿320 and ∿260 nucleotides, respectively. The longest of the three presumptive mRNAs would most likely code for the influenza B virus M polypeptide, while polypeptides corresponding to either of the shorter mRNAs have yet to be identified.

The NS_1 and NS_2 polypeptides do not share [^{35}S]-methionine-containing tryptic peptides, and the size of the NS_1 polypeptide is such that the nucleotide sequences coding for it must occupy almost the entirety of genome segment 8. Thus the sequences coding for most, if not all, of the NS_2 polypeptide must overlap with those coding for NS_1 and be translated in a different reading frame (34). The results suggest that the proportion of the NS_1 mRNA that is translated and the extent of the overlap between the NS_1 and NS_2 genes are greater in influenza B virus than was found previously with influenza A virus.

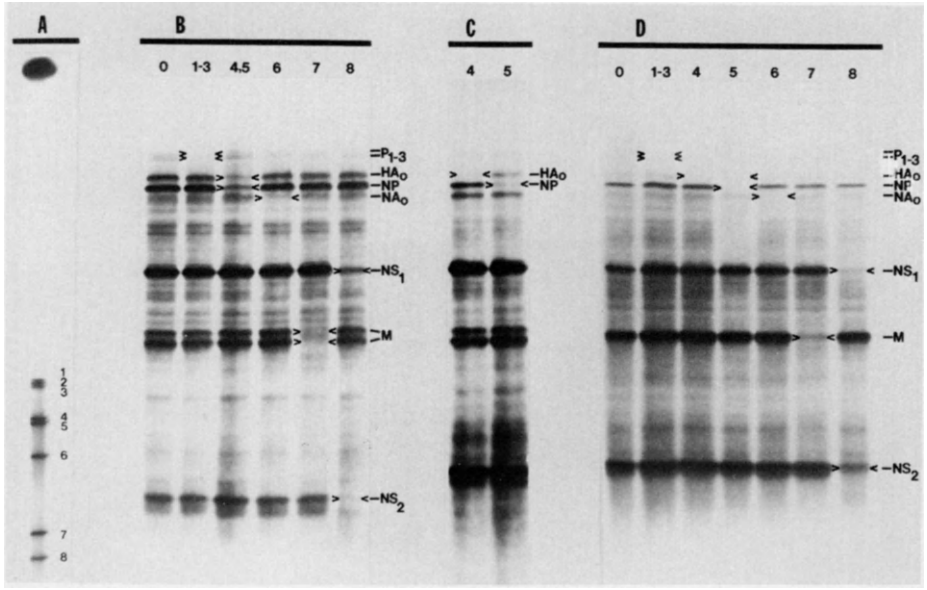

FIGURE 2. (A) Separation of [^{125}I]-labeled segments of influenza B/Lee/40 virus genome RNA on a 4% polyacrylamide/6M urea gel. (B) translation *in vitro* of total virus-specific mRNA after hybridization to individual genome RNA segments. A wheat germ extract was used without nuclease treatment. Lanes 1-3, 4-5, 6, 7, and 8 show polypeptides synthesized after hybridization of the respective genome RNA segments to mRNA. Lane 0 shows polypeptides synthesized from mRNA without addition of genome RNA. (C) as in B, but using separated genome segments 4 and 5. (D) as in B, but using wheat germ extract pretreated with *S. aureus* nuclease. Genome segment 5 was contaminated with genome segment 4 in this experiment. [From Briedis *et al.* (34).]

We have observed that the influenza B virus M polypeptide may migrate as a doublet on polyacrylamide gels, but that neither of the two M polypeptides was phosphorylated [a situation found with the Sendai virus M polypeptide (35)]. A possible explanation for this may be that the larger of the two polypeptides is a 'readthrough' protein caused by suppression of the normal termination codon by one of the suppressor tRNAs reported to be present in eucaryotic cells (36). Such a readthrough protein has been detected in tobacco plants infected by tobacco mosaic virus (37). This might also explain why nuclease treatment of wheat germ extracts abolished translation of the second M polypeptide, since such treatment might preferentially degrade suppressor tRNAs which were present in low concentration or were especially sensitive to *S. aureus* nuclease.

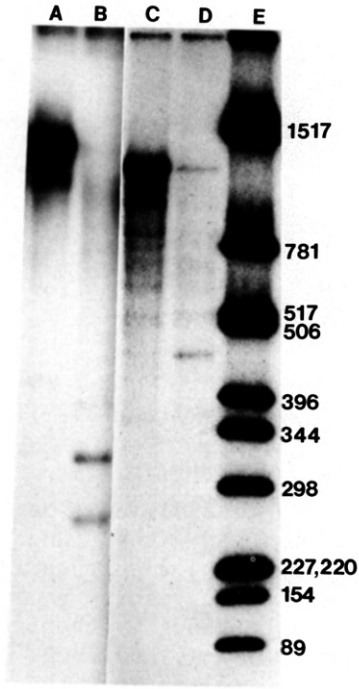

FIGURE 3. Analysis of influenza B virus-specific vRNA-mRNA hybrids. [^{125}I]-labeled influenza B virus RNA segments 7 and 8 were each hybridized to high and low molecular weight fractions of gradient fractionated total virus-specific mRNA and then treated with ribonucleases A, T_1, and T_2, before analysis on a 10% polyacrylamide gel buffered with SDS/glycine. When translated in the wheat germ cell-free system, the high molecular weight gradient fraction produced mainly M and NS_1 polypeptides, while the low molecular weight fraction produced mainly NS_2 polypeptide. (A) RNA segment 7 hybridized to high molecular weight mRNA. (B) RNA segment 7 hybridized to low molecular weight mRNA. (C) RNA segment 8 hybridized to high molecular weight mRNA. (D) RNA segment 8 hybridized to low molecular weight mRNA. (E) ^{32}P-labeled double-stranded DNA size markers. Lengths noted are in nucleotide base-pairs.

We are presently determining the nucleotide sequence of influenza B virus genome segment 8 by analysis of cloned cDNAs to determine the exact nucleotides that are used for translation of NS_1 and NS_2 and we are also determining the sequence of the NS_2 mRNA. The limited sequence data available suggests that the nucleotide sequences of RNA segment 8 of A and B viruses are totally different (38). It will be of great interest to investigate whether the interrupted regions and arrangement of the A viruses NS_2 mRNA is retained in B viruses.

III. MESSENGER RNAs AND PROTEINS DERIVED FROM INFLUENZA A VIRUS RNA SEGMENT 7

A. Conservation of the Influenza A Virus Membrane Protein (M_1) Amino Acid Sequence and an Open Reading Frame of RNA Segment 7 Encoding a Second Protein (M_2) in H1N1 and H3N2 Strains

Recently, the sequences of RNA segment 7 of two different passage lines of the A/PR/8/34 strain of influenza virus, which codes for the membrane protein were determined (39, 40). The cloned DNA sequence predicted one open reading frame that could code for a polypeptide of the size of the M_1 polypeptide (Mr $\sim$28,000), and a second open reading frame, overlapping the first by 68 nucleotides, that could code for a polypeptide of 97 amino acids. As a second protein product coded by influenza virus RNA segment 7 had not been reported, we sequenced RNA segment 7 of influenza strain A/Udorn/72 and compared this sequence to that obtained from the A/PR/8/34 strain, which had been isolated 38 years earlier, to establish whether the second open reading frame had been conserved.

A clone (pFV45/M) derived from annealed cDNA copies of A/Udorn/72 RNA segment 7 and its large mRNA (M DNA) cloned in the single Pst 1 site of plasmid pBR322 had been described previously (21). The M DNA was sequenced and found to contain a full sequence representation of the A/Udorn/72 RNA segment 7 (41). The second open reading frame of 97 amino acids was found to be conserved between the two strains, suggesting that it may code for a polypeptide. In addition, the other striking feature of the comparison of sequences of the segment 7 RNAs of the Udorn and PR8 strains is their similarity (41). There are only 42 nucleotide changes resulting in only 7 amino acid changes in the M_1 protein, and only one of these involves a change in charge (Asp to Asn). The long hydrophobic sequence from amino acid 115 to 151, containing only two charged

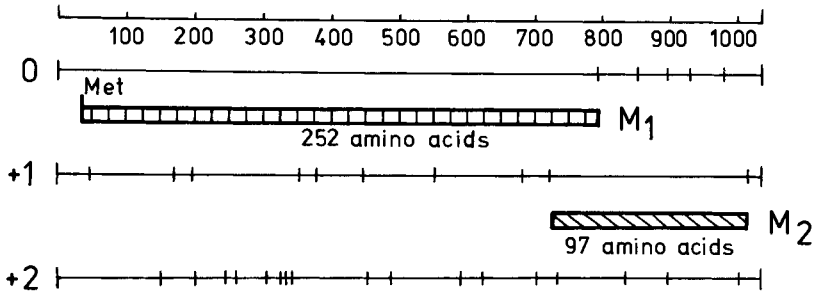

FIGURE 4. Schematic representation of the termination codons in the cloned M DNA using all three possible reading frames. The termination codons in all three possible reading frames (0, +1, +2) are denoted by a vertical line. The open reading frames coding for proteins M_1 and M_2 are shown as cross-hatched rectangles. [From Lamb and Lai (41).]

amino acids, is unchanged except for Ala to a Ser. In contrast to these results with M_1, in the NS_1 coding region of RNA segment 8, which is smaller in size, there are 78 nucleotide changes leading to 26 amino acid changes between Udorn and PR8 (19, 42). These observations suggest that the sequence of the M_1 protein is conserved between strains, a finding supported by the inability to distinguish the M_1 proteins serologically (43), and the lack of distinguishable characteristics in peptide maps of the M_1 protein between many human and avian strains (44). The paucity of changes in the amino acid sequences of the M_1 proteins is somewhat at variance with the assumed differences that were based on nucleotide homology using hybridization techniques (5). The open reading frames are shown schematically in Fig. 4.

B. Identification of a Second Protein (M_2) Encoded by RNA Segment 7 of Influenza A Virus

On re-examination of many autoradiographs of earlier gels of [^{35}S]-methionine-labeled infected cell lysates, a faint virus-specific polypeptide of Mr ∿15,000 could be detected, which had not been identified previously (e.g., see Fig. 5, Lamb and Choppin, ref. 15) and which migrated more slowly than NS_2. It seemed likely that this ∿15,000 Mr polypeptide could be the M_2 protein. Because the nucleotide sequence predicts that the M_2 protein contains 4 cysteine residues, cells were infected with various strains of influenza virus and labeled with [^{35}S]-cysteine in order to identify this protein with certainty (45). As shown in Fig. 5 a polypeptide of ∿15,000 Mr, designated M_2 could be readily detected. Its mobility varied amont strains, indicating that it is virus coded, and its mobility corresponds to the faint polypeptide present in the earlier [^{35}S]-methionine-labeled gels mentioned above. Polypeptide NS_2 cannot be observed in Fig. 5, which is expected because the nucleotide sequence predicts that it does not contain cysteine (19).

Protein M_2 was found to be translated from a distinct small mRNA (45) by separating on sucrose gradients, polyadenylated mRNAs isolated from influenza virus infected cells and translating each fraction *in vitro* using wheat germ extracts and [^{35}S]-cysteine as a labeled precursor.

Protein M_2 was demonstrated to be coded for by RNA segment 7 by using the RNA:RNA hybrid-arrested translation method and [^{35}S]-cysteine as labeled precursor (45). This coding assignment was confirmed by a genetic approach using

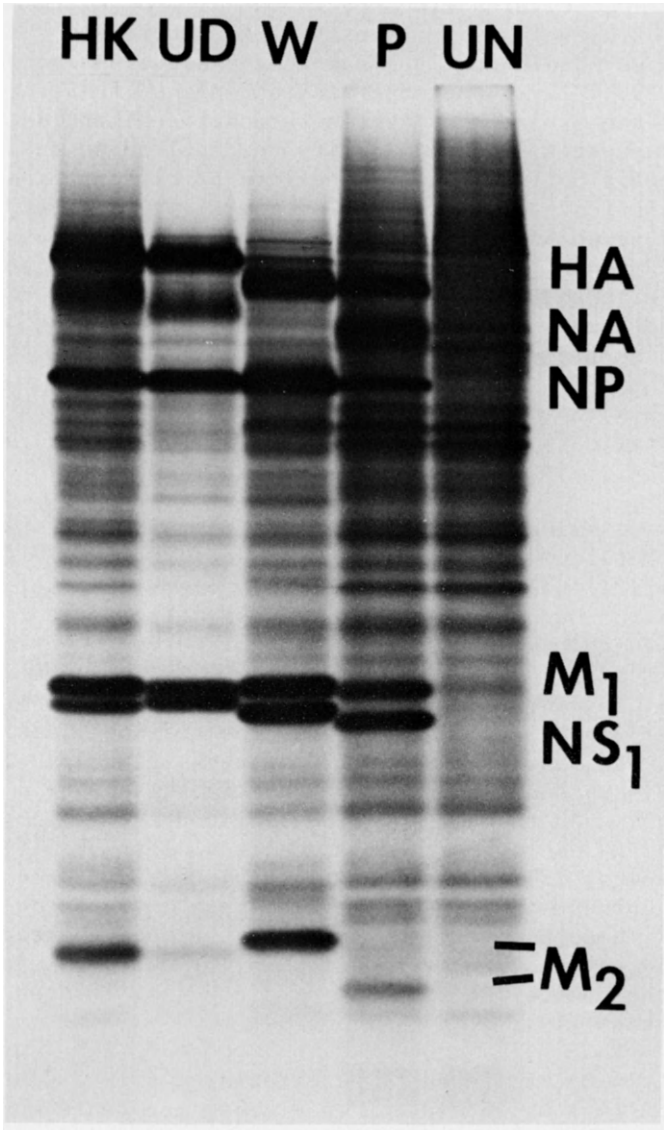

FIGURE 5. Identification of the M_2 polypeptide in HeLa cells infected with various strains of influenza A virus. 5 hr after infection, cells were labeled with [^{35}S]-cysteine (50 μCi/ml, 1200 Ci/m mole) for 1 hr and subjected to electrophoresis on a 17.5% acrylamide /4M area gel. The strains used were Hong Kong /68(HK), Udorn/72(UD), WSN /33(W) and PR/8/34 (P). U, uninfected cells. [From Lamb and Choppin (45).]

recombinants of PR/8 and HK containing different RNA segments of defined parental origin (kindly made available by Drs. Schulman and Palese) which we had also used earlier to define the gene coding for NS_2 (15). Because of the polyacrylamide gel electrophoretic mobility difference between the M_2 polypeptide of PR/8 and HK the coding assignment of M_1 and M_2 on RNA segment 7 could be readily deduced (45).

Peptide mapping of [^{35}S]- methionine or [^{35}S]-cysteine labeled M_1 and M_2 polypeptides showed that they are distinct polypeptides and in addition the maps could be correlated with the two open reading frames on RNA segment 7 (45).

Protein M_2 does not become incorporated into virus particles and is thus another nonstructural protein but its role in virus replication, like that of NS_1 and NS_2, remains to be established.

C. Sequences of mRNAs Derived from RNA Segment 7 of Influenza A Virus: Colinear and Interrupted mRNAs Code for Overlapping Proteins

Sequencing of the M_2 mRNA by the primer extension method has demonstrated that it contains an interrupted sequence of 680 nucleotides (46). The ∿51 virus-specific nucleotides comprising the 5'-end leader sequence of the M_2 mRNA are the same as those found at the 5'-end of the colinear M_1 mRNA. Following the leader sequence of the M_2 mRNA there is a 271 nucleotide body region that is 3'-co-terminal with the M_1 mRNA. Another small potential mRNA ($mRNA_3$) related to RNA segment 7 has also been found (46). This $mRNA_3$ has a leader sequence of ∿11 virus-specific nucleotides that are the same as the 5'-end of the M_1 and M_2 mRNAs, followed by an interrupted sequence of 729 nucleotides, and then a body region of ∿271 nucleotides that is the same as that of the M_2 mRNA. The nucleotide sequences found at the junctions of the interrupted sequences in M_2 mRNA and $mRNA_3$ are similar to those found at the splicing points of intervening sequences in eucaryotic mRNAs (24-26) but cannot be fitted to more complicated "consensus" sequences involving complementarity with the U1 snRNA (47, 48). Both the M_2 mRNA and $mRNA_3$ contain 10-15 heterogeneous non-viral nucleotides at their 5'-ends which appear to be derived from cellular RNAs used for priming the transcription of viral RNAs. Our findings showed that there is predominantly, but not exclusively an A residue at the position in the mRNA corresponding to the 3'-terminal U residue of the virion strand. This finding is in contrast to the complete

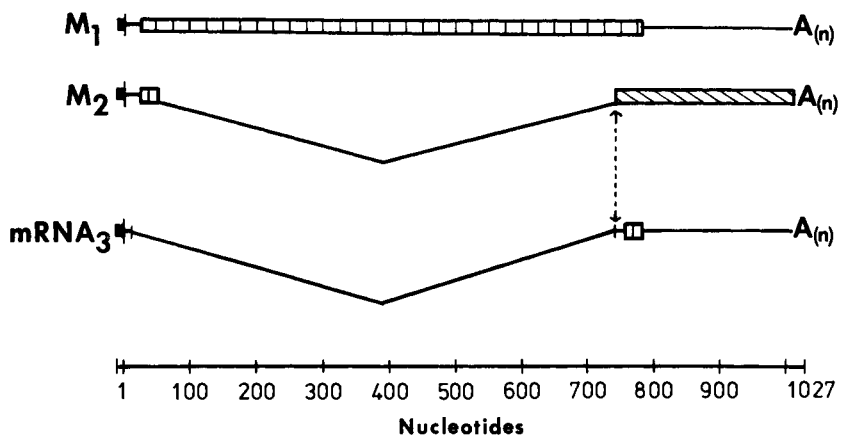

FIGURE 6. Model for the arrangement of the M_1, M_2 mRNAs & $mRNA_3$ and their coding regions. The thin lines at the 5'- and 3'-termini of the mRNAs represent the non-coding regions. The cross-hatched areas represent the coding regions of the mRNAs. In the region 740-1004 the M_2 mRNA is translated in a reading frame different from that used for M_1. No evidence has yet been obtained that $mRNA_3$ is translated, but its coding potential (which corresponds to the carboxyl terminal region of the M_1 protein) is indicated. The V-shaped lines represent the interrupted regions. The filled-in boxes before nucleotide 1 at the 5'-termini represent the heterogeneous nucleotides derived from cellular mRNAs that are covalently linked to the viral sequences. [From Lamb et al. (46).]

heterogeneity found by Caton and Robertson (49) but in support of our earlier observations (19). The small amount of heterogeneity at this position in a population of specific mRNAs for one RNA segment is also represented in the nucleotides found at position 1 in the cloned mRNAs (49, 50). This observation lends support to the theory that the virion transcriptase only elongates the primer starting at the 2nd nucleotide at the 3'-end of the virion strand (23, 49-51). We also observed an indication of a predominance of a GC dinucleotide in the heterogeneous nucleotides immediately before position 1, suggesting that among the primer RNAs used, there is a selection process favoring those having the sequence GCA immediately before the site of cleavage and elongation to form influenza virus mRNAs (46).

Because the 5'-end sequences of the M_1 mRNA and the M_2 mRNA are the same and share the 5'-proximal initiation codon for protein synthesis, the first 9 amino acids would be the same in the M_1 and M_2 proteins, and then the sequences diverge. The 271 nucleotide body region of the M_2 RNA can be translated in the +1 reading frame, and the sequence indicates that M_1 and M_2 overlap by 14 amino acids. Polypeptide M_2 has been estimated to have an apparent Mr ∿15,000 on polyacrylamide gels (46) but the sequence indicates it contains only 97 amino acids. Thus, the migration of the M_2 polypeptide on polyacrylamide gels, which differs among strains (46), is anomalous with respect to size and depends on amino acid composition. It should be noted that NS_2 was originally estimated to be Mr ∿11,000 but from the sequence analysis was found to be Mr 14,216 (19). Examination of the coding potential of $mRNA_3$ indicates that it would code for only nine amino acids (including the initiation methionine), assuming that the 5'-proximal initiation AUG codon is used for translation. These amino acids would be identical to the carboxyl terminal region of the M_1 protein. Whether this mRNA does code for such a peptide in infected cells or is a pseudo mRNA is a subject of our continuing investigation. A schematic arrangement of the mRNAs derived from RNA segment 7 and their coding regions is shown in Fig. 6. The sizes of the two additional small influenza B virus-specific mRNAs, whose presence was suggested by analysis of nuclease-treated vRNA-mRNA hybrids (Fig. 3) correspond very closely to those of influenza A M_2 RNA and $mRNA_3$.

Since we have shown that the M_2 mRNA, $mRNA_3$ and the NS_2 RNA have interrupted regions with junctions similar to those found in spliced eucaryotic mRNAs, it seems likely that these mRNAs are derived by splicing from their colinear transcripts,

M_1 and NS_1 mRNAs respectively. As discussed above for the NS_1 mRNA and must also be true for the M_2 mRNA and $mRNA_3$, the mechanism must be controlled as it has been found to be in the alternative splicing pathways to form different mRNAs from the colinear RNA transcripts of adeno virus and the papova viruses (reviewed in ref. 52). However, with influenza virus, the situation is slightly different because the presumed precursor RNAs also act as mRNAs from which polypeptides are translated (M_1 and NS_1).

ACKNOWLEDGMENTS

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REFERENCES

1. Palese, P., and Schulman, J.L., J. Virol. 17, 876 (1976).
2. Pons, M.W., Virology 69, 789 (1976).
3. McGeoch, D.J., Fellner, P., and Newton, C., Proc. Natl. Acad. Sci. USA 73, 3045 (1976).
4. Ritchey, M.B., Palese, P., and Schulamn, J.L., J. Virol. 20, 307 (1976).
5. Scholtissek, C., Harms, E., Rohde, W., Orlich, M., and Rott, R., Virology 74, 332 (1976).
6. Inglis, S.C., McGeoch, D.J., and Mahy, B.W.J., Virology 78, 522 (1977).
7. Lazarowitz, S.G., Compans, R.W., and Choppin, P.W., Virology 46, 830 (1971).
8. Skehel, J.J., Virology 49, 23 (1972).
9. Inglis, S.C., Carroll, A.R., Lamb, R.A., and Mahy, B.W.J., Virology 74, 489 (1976).
10. Lamb, R.A., and Choppin, P.W., Virology 74, 504 (1976).
11. Follett, E.A.C., Pringle, C.R., Wunner, W.H., and Skehel, J.J., J. Virol. 13, 394 (1974).
12. Minor, P.D., and Dimmock, N.J., Virology 67, 114 (1975).
13. Lamb, R.A., Etkind, P.R., and Choppin, P.W., Virology 91, 60 (1978).

14. Lamb, R.A., and Choppin, P.W., in "Negative Strand Viruses and the Host Cell" (B.W.J. Mahy and R.D. Barry, eds.), p. 229 Academic Press, London, (1978).
15. Lamb, R.A., and Choppin, P.W., Proc. Natl. Acad. Sci. USA 76, 4908 (1979).
16. Inglis, S.C., Barrett, T., Brown, C.M., and Almond, J.W., Proc. Natl. Acad. Sci. USA 76, 3790 (1979).
17. Sleigh, M.J., Both, G.W., and Brownlee, G.G., Nucl. Acids. Res. 6, 1309 (1979).
18. Lamb, R.A., Choppin, P.W., Chanock, R.M., and Lai, C-J., Proc. Natl. Acad. Sci. USA 77, 1857 (1980).
19. Lamb, R.A., and Lai, C-J., Cell 21, 475 (1980).
20. Robertson, J.S., Nucl. Acids. Res. 6, 3745 (1979).
21. Lai, C-J., Markoff, L.J., Zimmerman, S., Cohen, B., Berndt, J.A., and Chanock, R.M., Proc. Natl. Acad. Sci. USA 77, 210 (1980).
22. Krug, R.M., Broni, B.A., and Bouloy, M., Cell 18, 329 (1979).
23. Dhar, R., Chanock, R.M., and Lai, C-J., Cell 21, 495 (1980).
24. Breathnach, R., Benoist, C., O'Hare, K., Gannon, F., and Chambon, P., Proc. Natl. Acad. Sci. USA 75, 4853 (1978).
25. Seif, I., Khoury, G., and Dhar, R., Nucl. Acids Res. 6, 3387 (1979).
26. Lewin, B., Cell 22, 324 (1980).
27. Shaw, M.W., and Compans, R.W., J. Virol. 25, 608 (1978).
28. Kilbourne, E.D., ed., "The Influenza Viruses and Influenza" Academic Press, New York, (1975).
29. Ritchey, M.B., Palese, P., and Kilbourne, E.D., J. Virol. 18, 738 (1976).
30. Oxford, J.S., J. Virol. 12, 827 (1973).
31. Choppin, P.W., Lazarowitz, S.G., and Goldberg, A.R., in "Negative Strand Viruses" (R.D. Barry and B.W.J. Mahy, eds.), Vol. 1, p. 104 Academic Press, New York, (1975).
32. Almond, J.W., Haymerle, H.A., Felsenreich, V.D., and Reeve, P., J. Gen. Virol. 45, 611 (1979).
33. Racaniello, V.R., and Palese, P., J. Virol. 29, 361 (1979).
34. Briedis, D.J., Lamb, R.A., and Choppin, P.W., Virology (1981), in press.
35. Lamb, R.A., and Choppin, P.W., Virology 81, 382 (1977).
36. Geller A.I., and Rich, A., Nature 283, 41 (1980).
37. Pelham, H.R.B., Nature 272, 469 (1978).
38. Air, G.M., and Hackett, J.A., Virology 103, 291 (1980).
39. Winter, G., and Fields, S., Nucl. Acids. Res. 8, 1965 (1980).
40. Allen, H., McCauley, J., Waterfield, M., and Gething, M-J., Virology 107, 548 (1980).
41. Lamb, R.A., and Lai, C-J., Virology (1981), in press.

42. Baez, M., Taussig, R., Zazra, J.J., Young, J.F., Palese, P., Reisfeld, A., and Skalka, A.M., Nucl. Acids. Res. 8, 5845 (1980).
43. Oxford, J.S., and Schild, G.C., Virology 74, 394 (1976).
44. Dimmock, N.J., Carver, A.S., and Webster, R.G., Virology 103, 350 (1980).
45. Lamb, R.A., and Choppin, P.W., Virology (1981), in press.
46. Lamb, R.A., Lai, C-J., and Choppin, P.W., Proc. Natl. Acad. Sci. USA (1981), in press.
47. Lerner, M.R., Boyle, J.A., Mount, S.M., Wolin, S.L., and Steitz, J.A., Nature 283, 220 (1980).
48. Rogers, J., and Wall, R., Proc. Natl. Acad. Sci. USA 77, 1877 (1980).
49. Caton, A.J., and Robertson, J.S., Nucl. Acids. Res. 8, 2591 (1980).
50. Robertson, H.D., Dickson, E., Plotch, S.J., and Krug, R.M., Nucl. Acids. Res. 8, 925 (1980).
51. Bouloy, M., Plotch, S.J., and Krug, R.M., Proc. Natl. Acad. Sci. USA 77, 3952 (1980).
52. Ziff, E.B., Nature 287, 491 (1980).

STRUCTURE OF THE HEMAGGLUTININ GENE OF H0N1 STRAIN OF HUMAN INFLUENZA VIRUS AND ITS EXPRESSION IN *ESCHERICHIA COLI*

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ABSTRACT

The structure of the hemagglutinin gene of the H0N1 strain of human influenza virus has been compared to those of other strains. It was found that the H0 hemagglutinin showed the highest homology to the H2 hemagglutinin, suggesting a close geneological relationship between the two.

To express the H0 viral HA in *Escherichia coli* it was placed under control of the *lac* operon of *E. coli*. To achieve a high level of expression it was necessary to fuse the H0 hemagglutinin to the *E. coli* protein β-galactosidase. Fused proteins containing selected portions of HA were specifically precipitated by anti WSN antiserum and therefore, contained antigenic determinants.

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INTRODUCTION

Influenza viral hemagglutinin (HA) is a surface glycoprotein with a molecular weight of 75,000-79,000 and forms trimeric protein spikes on the viral surface coat. The protein is synthesized as a single polypeptide, glycosylated, and later cleaved at an internal arginine to smaller glycoproteins termed HA1 (MW 47,000) and HA2 (MW 28,000-29,000). It has been shown that HA1 lies at the N-terminus of uncleaved HA and HA2 at the C-terminus (1). Several lines of evidence indicate that HA1 is the area of the HA molecule involved in antigenic variation. First, natural variants of antigenic drift and in vitro variants selected with monoclonal antibodies were found to possess changes in the amino acid sequence of HA1 (2-4). Antigenic variants selected with monoclonal antibodies show single amino acid substitutions in the sequence of the HA1 polypeptide. One variant of A/Mem/1/71 (H3N2) could be distinguished from parental virus with heterogenous antibodies and had a change at amino acid 144 of HA1. A similar change was found in naturally occurring variants at this residue (3). A tryptic peptide (amino acids 153-159, ref. 5, 6) has also been shown to be changed in several variants of H0N1 virus (A/PR8/34) selected with monoclonal antibodies (7). Second, in the H3 (A/Memphis/102/72) virus the large CNBr fragment of HA1 (amino acids 1-170) has been shown to be antigenic, whereas CNBr fragments of HA2 do not bind to antigen or elicit immune response (8, 9). Finally, four locations of antigenic sites have been proposed for the H3 hemagglutinin based upon its three dimensional structure. These include site A, amino acids 140-146; site B, amino acids 187-196; site C, a bulge in the structure between cys 52 and cys 277, and site D, a region in the interface of trimeric subunits (10, 11).

To study the structure of the H0 hemagglutinin we have recently cloned the HA gene of the A/WSN/33 strain (12) and completed the sequence of this gene (5, 6). In addition we have joined this HA to bacterial plasmids to elicit the expression of antigenic determinants of the viral HA.

Structure of H0 Hemagglutinin

The A/WSN HA gene is 1775 nucleotides in length coding for 565 amino acids. The cRNA contains a 5' non-coding region of 32 nucleotides, a coding region of 1695 nucleotides and a 3' non-coding region of 48 nucleotides. The sequence shows a 17 amino acid signal prepeptide at the amino terminus followed by HA1 (325 amino acids), a single

arginine connecting residue, and HA2 (222 amino acids) at the carboxy terminus. The overall structure of the H0 HA is similar to that found in other strains suggesting a basic overall architecture for the molecule. Table 1 shows a comparison of amino acid homologies of the H0 hemagglutinin to the hemagglutinins of several other strains. The H0 strain and the human H2 strain A/Japan/305/57 show a 67% overall amino acid homology, which is the highest among the subtypes, suggesting a close geneologic relationship between them (5,6). Similarly, the H0-H2 pairing also showed the highest nucleotide homology in the coding region among subtype pairing (5, 6).

TABLE 1

Intersubtypic Amino Acid Homologies

	H2 (A/Japan/57)	H3 (A/Memphis/72)	Havl (A/FPV/Rostock/34
H0 (A/WSN/33)	57,81,67[a]	35,52,42	35,51,41
Havl (A/FPV/Rostock/34)	36,53,43	37,66,49	
H3 (A/Memphis/72)	36,49,42		

[a]The numbers indicate the percent amino acid homology between indicated subtype pairings in the 1) HA1 chain (pos. 1-325), 2) HA chain (pos. 327-548), and 3) total (pos. 1-548). Homologies were calculated as numbers of homologous residues/ total number of pairings. Deletions were counted as mismatches unless they coincided. Coincident deletions were not counted (taken from Hiti, Davis, and Nayak, Virology, in press).

Plasmids Containing Fusions of HA DNA and β-galactosidase DNA

To express HA DNA efficiently in E. coli it was necessary to fuse the DNA in the correct reading phase to the DNA coding for the first 1006 amino acids of β-galactosidase. Pre-sequences coding for the untranslated region at the 5' end of vRNA and the prepeptide were first removed by a modification of the procedure of Goeddel, et al. (13),

utilizing a primer complementary to first 16 nucleotides of the mature HA sequence. The modification included the addition of a methionine codon before the primer. Fig. 1 shows two of the final plasmid constructions.

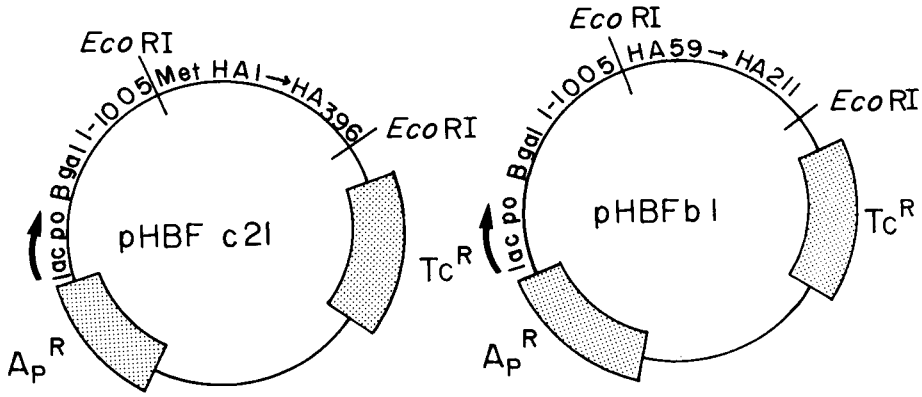


Figure 1. Plasmid construction for the expression of HA antigenic determinants.

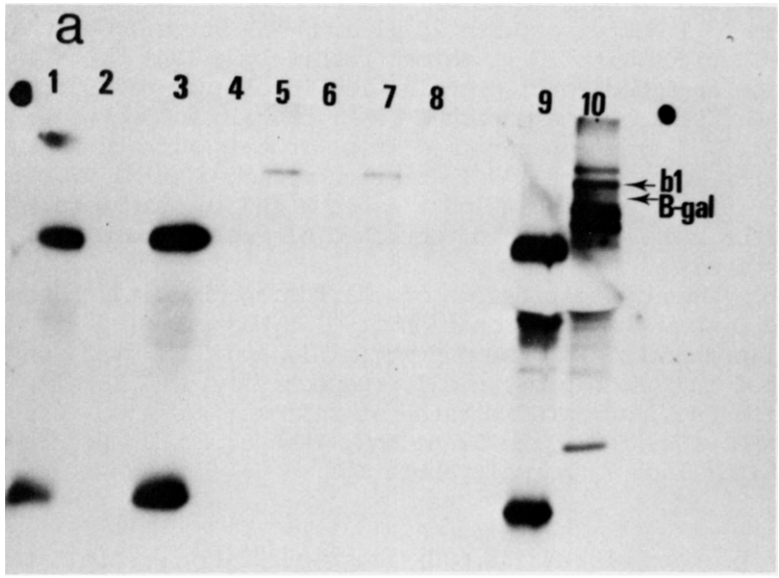
pHBFc21 contains DNA coding for amino acids 1006 of β-galactosidase fused to DNA coding for amino acids 1-396 of HA and pHBFb1 contains DNA coding for amino acids 59-211 of HA fused to β-galactosidase DNA. Each plasmid contains the lac promoter and operator.

Characterization of Fusion Proteins Containing HA Sequences

When total cell lysates of E. coli containing plasmids pHBFb1 or pHBFc21 were examined on SDS-polyacrylamide gels (14) both exhibited new proteins of the size expected for the DNA fusions described above. The production of these proteins was enhanced by the inducer IPTG, demonstrating their control by the lac operon. In addition they were present in the insoluble membrane fraction of E. coli lysates.

To study the immunological properties of these proteins they were partially purified by the procedure of Wetzel, et al. (15) and radioiodinated. Fig. 2a and 2b show the

immunoprecipitation (16) of enriched fractions containing fusion proteins c21 and b1 using anti WSN IgG.



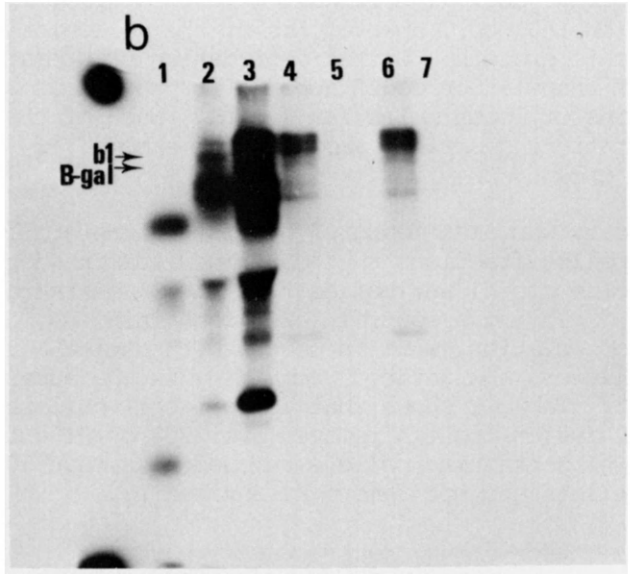


FIGURE 2 (Legend on following page)

Figure 2. a) Immunoprecipitation of b1 fusion protein. Lane 1, ^{125}I WSN virus with 10 µl anti-WSN virus IgG showing specific immunoprecipitation of viral HA (uncleaved top band) and M (bottom band). Lane 2, ^{125}I WSN virus without IgG; lane 3, ^{125}I WSN virus with 20 µl anti-WSN virus IgG; lane 4, ^{125}I WSN virus with 20 µl normal rabbit IgG; lane 5, ^{125}I fraction enriched in b1 protein with 10 µl anti-WSN virus IgG; lane 6, ^{125}I fraction enriched in b1 protein without IgG; lane 7, ^{125}I fraction enriched in b1 protein with 20 µl anti-WSN virus IgG; lane 8, ^{125}I fraction enriched in b1 protein with 20 µl normal rabbit IgG. Lanes 9 and 10 show total ^{125}I WSN virus and ^{125}I fraction enriched b1 fusion protein, respectively.

b) Immunoprecipitation of c21 fusion protein. Lanes 1, 2, and 3 show the fractionation of ^{125}I WSN virus, ^{125}I fraction enriched in fusion protein b1, and ^{125}I fraction enriched in fusion protein c21, respectively. ^{125}I c21 fraction was immunoprecipitated as follows: Lane 4, 10 µl anti-WSN virus IgG; lane 5, no IgG, lane 6, 20 µl anti-WSN virus IgG; lane 7, normal rabbit IgG.

Fusion protein b1 (MW 131,600) is specifically precipitated by anti-WSN antiserum (Fig. 2a) whereas a number of c21 (MW 130,000-160,000) fusion proteins are precipitated by anti-WSN antiserum (Fig. 2b). The largest c21 fusion protein precipitated (MW 160,000), however, is of the molecular weight expected for this protein. Either degradation or premature termination of translation could account for the immunoprecipitable proteins of lower molecular weight. Each of the immunoprecipitations described above could be competed completely by excess WSN virus.

Antigenicity was also measured by testing the ability of *E. coli* cell pellet fractions that contained fusion proteins b1 and c21, to remove HI antibodies from rabbit antisera. Cell pellets, including a control (c20) containing HA DNA in incorrect orientation, were incubated with anti-WSN rabbit antiserum, pelleted, and antibody remaining in the supernatant measured by HI. Table 2 shows that *E. coli* cell pellets containing c21 fusion protein removed over 90% of HI antibodies, while b1 containing pellets removed less than 50%, and control pellets did not remove HI antibodies.

TABLE 2

Adsorption of Anti-WSN Virus IgG with c2l and bl Fusion Proteins

Treatment	HI Titer[1] Exp. I	Exp. II
No adsorption	512	128
Adsorbed with E. coli 294/pHBFc20 cell pellet		
10 µl	512	-
100 µl	256	-
1000 µl	512	128
Adsorbed with E. coli 294/pHBFbl cell pellet		
10 µl	512	-
100 µl	512	-
1000 µl	256	64
Adsorbed with E. coli 294/pHBFc2l cell pellet		
10 µl	512	-
100 µl	128	-
1000 µl	8	8

[1]HI titer was measured in duplicate by the microtest system.
[2]Cell pellets were prepared from 1 liter culture and resuspended in 6 ml PBS. The volume of cell pellet shown was incubated with either 512 (Exp. 1) or 128 (Exp. II) HI units anti-WSN virus IgG, the mixture of IgG and cell pellet centrifuged, and the HI activity remaining in the supernatant measured.

This result indicates that c2l protein contains more antigenic determinants than bl, as expected.

DISCUSSION

We describe here the expression of proteins in E. coli that contain functional antigenic determinants of influenza viral hemagglutinin. One difference between this and previous studies (17) of HA expression in bacteria is that

presequences of HA have been removed from the HA DNA by use of a synthetic primer.

In pHBFb1, c21 and other plasmid constructions containing HA DNA (unpublished results) it has been found that different portions of HA can be expressed independently in E. coli by the techniques described above. These techniques therefore allow independent study of each of the proposed antigenic sites (10, 11), for example allowing determination of the boundary of the site and the effect of other amino acids removed from the site.

The expression of HA antigenic determinants in E. coli will also allow a test of the potential of such proteins as vaccines. Since these proteins are non-glycosylated will they elicit new antibodies to newly exposed sites on the molecule? Are there common antigenic determinants among the subtypes? The availability of such proteins will allow these and other questions to now be answered.

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REFERENCES

1. Skehel, J.J. and Waterfield, M.D. (1975). Proc. Natl. Acad. Sci. USA 72,93.
2. Laver, W.G., Air, G.M., Webster, R.G., Gerhard, W.,Ward, C.W. and Dopheide, T.A.A. (1979). 98,226.
3. Webster, R.G. and Laver, W.G. (1980). Virol. 104,139.
4. Laver, W.G., Air, G.M., Webster, R.G., Gerhard, W.,Ward, C.W. and Dolpheide, T.A. (1980). In: Structure and Variation in Influenza Virus (eds. Laver, W.G. and Air, G.M.), Elsevier, North Holland, pp. 295-306.
5. Hiti, A.L., Davis, A.R. and Nayak, D.P. (1981). Virol. in press.
6. Hiti, A.L., Davis, A.R. and Nayak, D.P. (1980). In: Negative Strand Viruses, in press.
7. Laver, W.G., Gerhard, W., Webster, R.G., Frankel, M.E.

and Air,G.M.(1979). Proc. Natl. Acad. Sci.USA 76,1425.
8. Jackson, D.C., Russell, R.J., Ward, C.W. and Dopheide, T.A. (1978). Virol. 89,199.
9. Jackson, D.C., Dopheide, T.A., Russell, R.J., White, D.O. and Ward, C.W. (1979). Virol. 93,458.
10. Wilson, I.A., Skehel, J.J. and Wiley, D.C. (1981). Nature 289,366.
11. Wiley, D.C., Wilson, I.A. and Skehel, J.J. (1981). Nature 289,373.
12. Davis, A.R., Hiti, A.L. and Nayak, D.P. (1980). Gene 10,205.
13. Goeddel, D.V., Sehpard, H.M., Yelverton, E., Leung, D. and Crea, R. (1980). Nucleic Acids Res. 8,4057.
14. Laemmli, U.K. (1970). Nature 227,680.
15. Wetzel, R., Heyneker, H.L., Goeddel, D.V., Jhurani, P., Shapiro, J., Crea, R., Low, T.L.K., McClure, J.E. and Goldstein, A.L. (1980). Biochemistry 19,6096.
16. Lamb, R.A., Etkind, P.R. and Choppin, P. (1978). Virol. 91,60.
17. Emtage, J.S., Tacon, W.C.A., Catlin, G.H., Jenkins, B., Porter, A.G. and Carey, N.H. (1980). Nature 283,171.

NONVIRAL SEQUENCES IN COMPLETE INFLUENZA VIRAL DNA CLONES AND FUNCTIONAL EXPRESSION OF CLONED DNA CODING FOR THE HEMAGGLUTININ

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SUMMARY

We prepared influenza viral DNA duplexes from cDNA copies by reverse-transcription of cytoplasmic viral mRNA and genomic viral RNA. DNA sequences corresponding to the gene segments that code for the nonstructural proteins, the matrix protein, the hemagglutinin, the neuraminidase and the nucleoprotein of influenza virus [strain A/Udorn/72 (H3N2)] were cloned in E. coli pBR322. Analysis of terminal nucleotides of several independently isolated viral DNA clones revealed that additional oligonucleotides were present at the 5' terminus of viral mRNA transcripts. The sequences of these additional nucleotides varied among DNA clones of the same gene and among DNA clones from different genes. The heterogeneity of these oligonucleotides suggests that they are derived from cellular RNA molecules. These results provide evidence that cellular RNA sequences are utilized to prime influenza viral mRNA transcription in virus-infected cells.

Sequence analysis also showed that sequences at both termini of vRNA were fully represented in a majority of cloned DNA segments. These cloned DNAs, therefore, could potentially produce full-length copies of viral RNA that contain all control signals for viral gene expression in eukaryotic cells. As a first step, we attempted to produce influenza viral polypeptides from the cloned DNA. Cloned complete DNA sequences coding for the hemagglutinin of influenza virus were inserted into the late region of a viable deletion mutant of SV40. The hybrid DNA was cloned and propagated in the presence of a ts A SV40 helper. Infection of primate cells with the hybrid virus produced a protein similar to the hemagglutinin of influenza virus as detected by immunoprecipitation and gel electrophoresis.

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The polypeptide product was glycosylated as revealed by incorporation of radioactive labelled sugars. In addition, the putative HA product exhibited functional activity as indicated by agglutination of red blood cells.

INTRODUCTION

The genome of influenza A virus consists of eight, single-stranded RNA segments ranging from 900 to 2400 nucleotides (1,2,3). These virion RNAs are templates for transcription of polyadenylated mRNA species coding for the specific viral polypeptides and functions in infected cells (4,5,6). There are seven viral coded proteins present in the virion including the 3 polymerase proteins, the hemagglutinin and neuraminidase surface glycoproteins, the nucleoprotein and the matrix protein (4,7). In addition, the two overlapping nonstructural proteins found inside the infected cells are encoded by gene 8, the smallest RNA segment (8,9). How these viral proteins regulate macromolecular synthesis and interact among themselves to form mature progeny virions in infected cells is only partially understood. Important questions concerning influenza viruses such as the origin and nature of genetic variability and the genetic basis for virulence in animals or birds remain to be answered. In order to investigate these questions and to better understand influenza viruses, we have recently obtained and characterized influenza viral DNA clones derived from cDNA copies of virion RNA and mRNAs (15). The complete cloned DNA sequences coding for the nonstructural proteins and for the matrix protein were determined and comparison of these nucleotide sequences with those obtained by others for different influenza viral subtypes indicated that the amino acid sequences for the two nonstructural proteins and the matrix protein were greatly conserved except for changes in a few amino acids (10,11,12,13,14).

Our influenza viral DNA was also employed to map the mRNA transcripts on their template RNA segments and to further sequence these mRNA molecules. These results indicate that two separate mRNAs, NS_1 mRNA and NS_2 mRNA are derived from RNA segment 8. Sequencing studies demonstrate that NS_1 mRNA is a co-linear transcript and the NS_2 mRNA contains an interrupted region similar to the intervening sequences of eukaryotic genes (11). Interrupted and uninterrupted mRNAs derived from RNA segment 7 coding for the matrix protein were also found (Lamb et al., this volume). In this report, we summarize findings of nonviral sequences in cloned full-length DNA fragments derived from cDNA copies

of genomic RNA and mRNA. Furthermore, we describe an approach using an SV40 vector to achieve functional expression in mammalian cells of cloned influenza DNA coding for the hemagglutin (HA).

MATERIALS AND METHODS

1. Viral Strains and DNA Cloning

Influenza virus A/Udorn/72 (H3N2) was used to derive DNA clones. Cloning procedures were described earlier (15).

2. Construction of HA-SV40 Virus

To facilitate packaging of complete cloned HA DNA in the SV40 virion, we used a viable deletion mutant of SV40, dl-2330, (16). Form I DNA from dl-2330 was cleaved with HaeII and Bam Hl and the large DNA fragment (fragment A) was isolated. HA specific DNA fragment was obtained from PstI digestion of pFV88, a hybrid molecule containing pBR 322 and DNA sequences corresponding to the complete HA gene. Both the SV40 A fragment (5 µg) and HA DNA (10 µg) were mixed and digested with S_1 nuclease at 45°C for 30 minutes. After phenol extraction and alcohol precipitation, the DNA components were dissolved in 100 µl of ligase reaction buffer containing 70 mM Tris, pH 7.6, 10 mM Mg Cl_2, 1mM ATP, 15 mM DTT, 20 µg/ml gelatin, and 5 units of ligase. The ligase reaction was carried out at 4°C overnight. Procedures for coinfection of African green monkey kidney (AGMK) cells with DNA products from the ligase reaction and with a tsA28 SV40 helper were essentially similar to the infectious center assay procedure (17).

3. Screening for HA-SV40 Hybrid Virus:

Viral plaques were isolated and virus stocks prepared from plaque isolates after infection of AGMK cells. Form I DNA was extracted from AGMK cells infected with the virus stock according to the Hirt procedure (25). Viral DNA was separated on an agarose gel and blotted on nitrocellulose paper for hybridization with a ^{32}P-labelled cDNA probe derived from reverse-transcription of influenza vRNA using the Southern blotting technique (18).

4. Polypeptide Labelling and SDS-Polyacrylamide Gel Electrophoresis

AGMK cells infected with influenza virus or HA-SV40 recombinant virus or uninfected control cells were incubated with ^{35}S-methionine (100 μCi/ml) in methionine free medium for 6 hours. RIPA buffer containing 1% triton, 1% deoxycholate, 0.15 M NaCl, 0.1% SDS, 0.1 M Tris, pH 7.5, 1mM PMSF and 5000 units trasylol was used to prepare cell lysates for immunoprecipitation with hemagglutinin antiserum. Protein A-sepharose (Pharmacia) was used to specifically bind the immune complex for analysis on SDS-polyacrylamide gel.

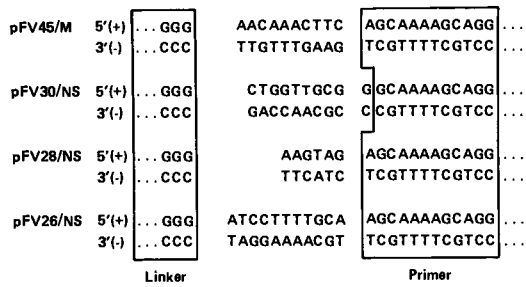
RESULTS:

1. Nonviral Sequences in Cloned Influenza Viral DNA

We described earlier a novel approach to obtain DNA clones containing sequences of influenza A viruses (15). The procedures involved separate synthesis of cDNA strands derived from genomic RNA and from cytoplasmic viral mRNA, construction of viral recombinant DNA duplexes and cloning of the DNA duplex in the E. coli-pBR322 system. This cloning approach also provided a strategy for analysis of additional 5'-terminal nucleotides in influenza viral mRNA species (19,20). Eight different DNA clones have been obtained and characterized. These clones include: plasmids pFV88 and pFV92 coding for the hemagglutinin; pFV101 and pFV102 coding for the neuraminidase; pFV45 coding for the matrix protein; and pFV26, pFV28, pFV30 coding for the nonstructural proteins. Analysis of the plasmid DNA by digestion with restriction enzymes followed by gel electrophoresis indicated that these clones contained complete or almost complete copies of cDNA transcripts. The results of sequence analysis of terminal nucleotides are shown in Figure 1A and Figure 1B. In each clone we observed a set of additional nucleotides was present between the oligo dG/dC linker and the dodecanucleotide primer. The sequences of these inserts varied in length from 6 to 14 nucleotides and were also heterogeneous in base composition among clones from the same gene and among clones from different genes. Several clones contained the entire dodecanucleotide primer sequences indicating that these cloned DNAs are complete copies of the 3' terminal nucleotides of genomic RNA.

Two of 8 clones (pFV101/NA and pFV30/NS) exhibited an alteration (A to G) of the first nucleotide of the primer.

(A)



(B)

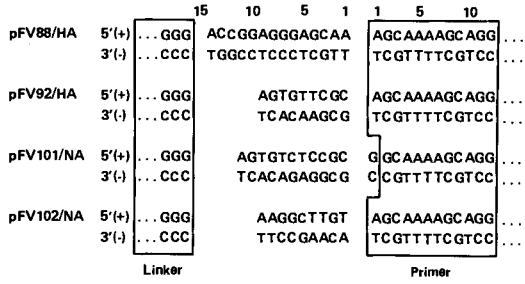


Figure 1. Heterogeneous 5'- terminal nucleotides of cloned influenza viral DNA.
(A) Shows the terminal sequences of cloned DNA coding for the matrix proteins (M) and the nonstructural proteins (NS) of influenza virus.
(B) Shows the terminal sequences of cloned DNA coding for the hemagglutinin (HA) and the neuraminidase (NA). The heterogeneous sequences between the linker and the primer were derived from cellular RNA covalently linked to the 5'- terminus of viral mRNA.

This frequent alteration suggests that a mechanism of mismatch correction occurred on the duplex DNA formed between cDNA copies and that the altered G residue was derived from cellular RNA sequences rather than from the viral RNA. This implies that transcription of influenza mRNA commences at the second nucleotide at the 3' terminus of vRNA. An AUG codon was not present in these RNA inserts indicating that these additional sequences probably do not play a significant role in mRNA translation. On the other hand, the

heterogeneity and the ubiquity of these inserts at the 5' terminus of mRNA suggest that these RNA inserts are derived from cellular RNA species and function in the initiation of mRNA transcription of viral RNA segments. These results provide direct evidence that cellular RNA molecules are utilized in priming viral mRNA transcription in infected cells. These observations confirm and extend the results of Krug and his colleagues (21,22) that eukaryotic mRNA species such as beta-globin mRNA can prime the transcriptase activity of influenza virions in vitro.

2. Full-length Viral Sequences in Cloned DNA Segments

Since we are interested in obtaining full-length DNA copies, the nucleotide sequences at the other terminus of the cloned DNA were also determined in order to insure their completeness and correctness. These results are presented in Figure 2.

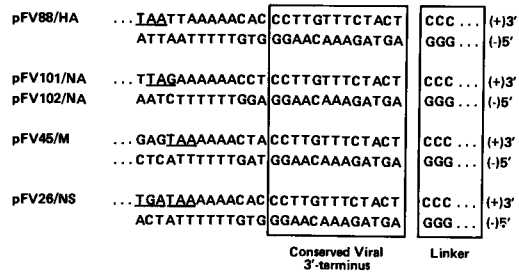


Figure 2. Complete 3'- terminal nucleotides of cloned influenza viral DNA. DNA clones that contain complete viral 3'- terminal sequences as indicated by full representation of all 13 conserved nucleotides of the viral terminus are shown.

As can be seen, these clones contained the entire 13 conserved nucleotides that are complementary to the 5' terminus of vRNA. The conserved viral sequences were followed immediately by an oligo dG/dC linker that was introduced during the construction of recombinant plasmid. Thus, at least one clone from each of the four cloned viral genes showed a complete representation of viral terminal sequences. Furthermore, the complete nucleotide sequence of pFV26 coding for the non-structural protein and, more recently, the entire sequence of pFV 45 coding for the matrix protein have been obtained. These result indicate that both pFV45 and pFV26 are full-length DNA copies of their genomic RNA (11,14).

3. Construction of HA-SV40 Hybrid DNA

SV40 vectors have been widely used for introduction of cloned DNA into mammalian cells to study gene expression (23,24). Cloned complete DNA coding for the hemagglutinin of influenza virus should be valuable in examining functional domains of the hemagglutinin molecules. From a consideration of packaging size in the SV40 virion, we chose a viable deletion mutant of SV40, (dl-2330), for insertion of the cloned full-length HA DNA in the late region. This mutant contains a deletion of approximately 240 nucleotides in the small t region and another 180 nucleotides in the late leader region of the SV40 genome (16). The procedures for construction of HA-SV40 DNA molecules and for further propagation of the recombinant virus in the presence of a ts A SV40 helper at a temperature restrictive for the helper (39°) are shown in Figure 3. The results of these experiments showed that 9 of 18 plaque isolates contained HA DNA sequences as detected by hybridization with a specific probe of ^{32}P-labelled cDNA derived from reverse-transcription of influenza vRNA.

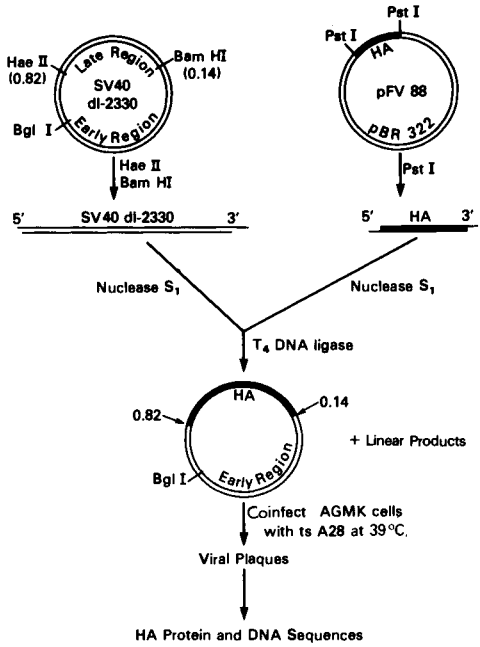


Figure 3. Procedures for construction of SV40-HA recombinant DNA.

4. Analysis of Hemagglutinin from Infection with HA-SV40 Hybrid Virus

Production of the hemagglutinin polypeptide was detected by labelling the HA-SV40 infected AGMK cells with ^{35}S-methionine. Labelled cell lysates were immunoprecipitated with hemagglutinin antiserum and analyzed by electrophoresis on SDS-polyacrylamide gels (see Materials and Methods). The analysis of immunoprecipitable proteins from infection with 9 HA-SV40 viral isolates are shown in Figure 4. Infection of AGMK cells with influenza virus in the absence of trypsin yielded predominantly an immunoprecipitible band of uncleaved hemagglutinin (HAo) of MW 70-75,000. There was also a small but detectable quantity of cleaved HA_1 and HA_2 of MW 45,000 and 30,000 respectively. After immunoprecipitation of cell lysates from hybrid virus infection, one isolate (gel track 8) produced a labelled band of MW 70-75,000 similar in size to the uncleaved hemagglutinin HAo of influenza virus. Other minor bands present in the same gel were non-specifically precipitated and therefore unrelated to the hemagglutinin since they were also found in uninfected cells.

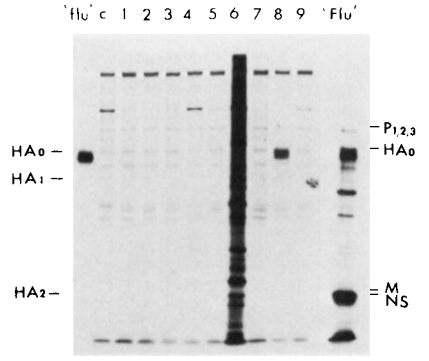


Figure 4. Analysis of the hemagglutinin polypeptide from infection with SV40-HA hybrid virus. Plaque isolates of SV40-HA hybrid virus were screened for the hemagglutinin production in infected cells. Cells were labelled with ^{35}S-methionine and lysates prepared in RIPA buffer were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. One through 9 = nine isolates of the hybrid virus screened. Virus isolate 8 produced a polypeptide similar to the hemagglutinin (HAo) of influenza virus. "Flu" and "flu" = lysates from infection with influenza virus before and after immunoprecipitation. C = lysates from uninfected cell control.

As can be seen from Figure 4, the HAo of influenza virus and the similar protein from infection with the HA-SV40 isolate showed a pattern of unusual broadness on SDS-polyacrylamide gel as compared with other polypeptide bands. This broad pattern suggests the polypeptide is highly glycosylated. In a separate experiment, infected AGMK cells were labelled with ^{3}H-mannose, ^{3}H-fucose and ^{3}H-N-acetylglucosamine for 2 hours and the lysates similarly analyzed as described. The results further confirm that the putative HAo from infection with HA-SV40 is glycosylated as revealed by incorporation of labelled sugars (data not shown).

5. Hemagglutination Activity of the Polypeptide Product from HA-SV40

To assay for functional hemagglutinin, we performed a hemagglutination test on the HA product from infection of AGMK cells with HA-SV40 recombinant. Guinea pig red blood cells were used for this assay. Lysates were prepared from uninfected control cells, from cells infected with the wild-type SV40 and from cells infected with the HA-SV40 hybrid virus and were serially diluted in a microwell plate for a standard hemagglutination assay with the red blood cells. The results are shown in Figure 5. It can be seen that the hemagglutination titer of lysates from HA-SV40 infection was higher than that of either control lysates.

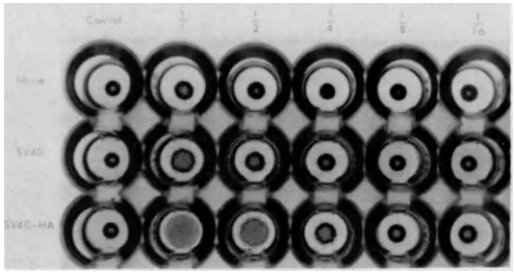


Figure 5. Hemagglutination activity of the hemagglutinin from infection with SV40-HA virus. Hemagglutination test using guinea pig red blood cells was carried out on cell lysates from (1) uninfected cell control, (2) infection with wild-type SV40, and (3) infection with SV40-HA hybrid virus. All lysates were serially diluted as indicated. Control = phosphate buffered saline alone and no lysates were added. Lysates from SV40-HA infection show higher hemagglutination activity than either control lysates.

In another experiment (data not presented) it was further demonstrated that the hemagglutination activity was readily inhibited by hemagglutinin antiserum indicating that the HA product from infection with HA-SV40 exhibits a hemagglutination function similar to the hemagglutinin of influenza virus.

DISCUSSION:

Several cloned DNA segments containing sequences from influenza genomic RNA and mRNA have been characterized by cleavage with restriction enzymes and by nucleotide sequence analysis. This analysis showed that these DNA clones represent complete copies of genomic RNA segments coding for the hemagglutinin, the neuraminidase, the matrix protein, and the nonstructural proteins (15). Additional nonviral sequences were deduced from each cloned DNA and the heterogeneity of these sequences among DNA clones suggests that cellular RNA molecules were utilized in initiation of viral mRNA transcription (19,20,22). Two of 8 clones contained an altered first viral nucleotide (A to G on the (+) DNA strand). This frequent alteration suggests that transcription of viral sequence commences with the penultimate nucleotide preceded by the nonviral sequences terminating with a G residue. If this is the case, the origin of an A residue at the first nucleotide of viral sequence in other clones cannot be determined since the dodecanucleotide primer used contains this A residue. One interpretation is that in this instance nonviral sequences containing a terminal A residue initiate viral mRNA transcription at the penultimate nucleotide. At any rate, these clones contained complete 3'-viral sequences. Analysis of nucleotides at the other terminus showed that complete 5'-viral sequences are also represented in each of the four viral genes examined. A complete sequence of pFV26/NS and pFV45/M recently obtained demonstrated conclusively that both clones are full-length copies of genomic RNA transcripts (11,14)

Full representation of viral genomic sequences in cloned DNA segments implies that these DNAs could potentially produce RNA copies that have all nucleotide signals for viral gene expression in eukaryotic cells. These DNA clones are therefore valuable for biological experiments, such as functional expression of polypeptides, and conversion of the cloned DNA back to virion RNA. Expression of functional polypeptides in mammalian cells would enable one to examine individual viral functions and their interactions. One could also dissect the functional domains of a

polypeptide at the DNA level. Furthermore, if the cloned DNA could be converted back to influenza virion RNA, one would be able to construct influenza mutants containing mutation sites at strategic locations. Such mutants should be valuable for biological studies as well as for use in immunoprophylaxis.

There are several advantages of using SV40 vectors for introduction of cloned DNA into mammalian cells: (a) the SV40 genome is small and well-characterized; (b) the replicon of SV40 provides for effective gene amplification in lytically infected cell, and (c) the SV40 genome contains both transcriptional and post-transcriptional signals to generate stable RNA species in the permissive AGMK cell system. To achieve functional expression of influenza DNA, we described an approach using such an SV40 vector. A hybrid HA-SV40 virus was constructed by insertion of the complete HA DNA into the late region of SV40. This hybrid was then propagated in AGMK cells using a helper ts mutant of SV40. One clone of HA-SV40 virus was capable of producing a polypeptide that was indistinguishable from the hemagglutinin of influenza virus by several criteria including immunoprecipitation by hemagglutinin antiserum, M.W. measurement by gel electrophoresis, glycosylation, and red blood cell agglutination activity.

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LITERATURE CITED

1. Palese, P. and Schulman, J.L. (1976). J. Virol. 17, 876-884.
2. Pons, M.W. (1976). Virology 69, 789-792.
3. McGeoch, D.J., Fellner, P. and Newton, C. (1976). Proc. Nat. Acad. Sci. USA 73, 3045-3049.
4. Ritchey, M.B., Palese, P. and Schulman, J.L. (1976). J. Virol. 20, 307-313.
5. Scholtissek, C., Harms, E., Rhode, W., Orlich, M. and Rott, R. (1976). Virology 74, 332-334.
6. Inglis, S.C., McGeoch, D.J., and Mahy, B.W.J. (1977). Virology 78, 522-536.

7. Skehel, J.J. (1972). Virology 49, 23-36.
8. Lamb, R.A. and Choppin, P.W. (1979). Proc. Nat. Acad. Sci. USA 76, 4908-4912.
9. Inglis, S.C., Barrett, T., Brown, C.M., and Almond, J.W. (1979). Proc. Nat. Acad. Sci. USA 76, 3790-3794.
10. Porter, A.G., Smith, J.C. and Emtage, J.S. (1980). Proc. Nat. Acad. Sci. USA 77, 5074-5078.
11. Lamb, R.A. and Lai, C.-J. (1980). Cell 211, 475-485.
12. Winter, G. and Fields, S. (1980). Nucleic Acids Res. 8, 1965-1975.
13. Allen, H., McCauley, J., Waterfield, M., and Gething, M.-J. (1980). Virology 107, 548-551.
14. Lamb, R.A. and Lai, C.-J. (1981). Virology, submitted.
15. Lai, C.-J., Markoff, L.J., Zimmerman, S., Cohen, S., Berndt, J.A., and Chanock, R.M. (1980). Proc. Nat. Acad. Sci. USA 77, 210-214.
16. Konig, M. and Lai, C-J. (1979). Virology 96, 277-280.
17. Lai, C.-J. and Nathans, D. (1974). J. Mol Biol. 89, 179-193.
18. Southern, E.M. (1975). J. Mol Biol. 98, 503-517.
19. Dhar, R., Chanock, R.M. and Lai, C-J. (1980). Cell 21, 495-500.
20. Caton, A.J. and Robertson, A.J. (1980). Nucleic Acids. Res. 8, 2591-2603.
21. Bouloy, M., Plotch, S.J. and Krug, R.M. (1978). Proc. Nat. Acad. Sci. USA 75, 4886-4890.
22. Krug, R.M., Broni, B.A., and Bouloy, M. (1979). Cell 18, 329-334.
23. Hamer, D. and Leder, P. (1979). Nature (London) 281, 35.
24. Mulligan, R.C. and Berg, P. (1980). Science 209, 1422-1427.
25. Hirt, B. (1967). J. Mol. Biol. 26, 365-369.

EXPRESSION OF THE INFLUENZA HEMAGGLUTININ USING SIMIAN VIRUS 40 VECTORS

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ABSTRACT

We have used deleted SV40 genomes that contain the entire early gene region, the origin of replication and late leader sequences to clone in monkey cells the hemagglutinin (HA) gene of influenza strain A/WSN. The HA gene (1775 base pairs long) was cloned in pBR322 using cDNA made from viral RNA (1). The cloned gene was further modified by treatment with nuclease Bal 31 to remove the dG:dC tails as well as some of the untranslated sequences, and recloned after addition of Bam H1 restriction endonuclease linkers. A number of SV40-HA recombinants were constructed by inserting recloned HA DNA into the late region of SV40. DNA sequencing indicated that the HA gene in different recombinants could be either expressed intact or as a fused protein from the VP2 locus of SV40. The SV40-HA recombinants complement in a lytic infection early deletion mutant helper viral genomes, and express the HA gene as detected by immunofluorescence and by immunoprecipitation of *in vivo* ^{35}S-labeled proteins.

INTRODUCTION

The genome of simian virus 40 (SV40) consists of a double-stranded circular DNA molecule of 5243 base pairs whose total sequence has been solved and whose genetic organization

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has been determined (2). Segments of this DNA have proved to be useful as recombinant DNA vectors for cloning and expressing a variety of genes in mammalian cells (3-5). Not only have small chromosomal genes with intervening sequences been shown to be functional in SV40 vectors, but also a number of structural genes derived from cDNA clones of mRNA species have been expressed efficiently using SV40 regulatory signals.

With the DNA cloning of the structural genes from RNA viruses such as influenza, the ability to examine the biological activity of both these sequences and specific alterations introduced into them becomes of importance. We have pursued this goal through the insertion of the complete coding DNA sequence for the influenza hemagglutinin into the late gene region of different SV40 vectors.

We describe here the construction, replication and expression of SV40-influenza hemagglutinin (strain A/WSN/33) recombinants. The hemagglutinin (HA) gene product is quantitatively the major surface glycoprotein of influenza virus (6) and the antigen against which neutralizing antibodies are directed (7,8). It is responsible for viral attachment to cell receptors (9) and functions in the early stages of infection (10,11). Uncontrolled human epidemics have been associated with extensive antigenic variation of the HA due to amino acid sequence changes (12,13).

RESULTS AND DISCUSSION

The SV40 Vectors

Construction of the SV40 vectors is described elsewhere (Hartman and Fareed, manuscript in preparation). Briefly two types of SV40 vectors were used (Figure 1). One (SVE3) consists of the fragment spanning the Bam Hl to Hpa II sites (clockwise on the SV40 map) thus containing the entire early region, the origin of replication and the initiation and termination signals for late gene transcription but lacking most of the acceptor sites for splicing and the bodies of the late gene products. The second (SVELL9) was derived from the above by addition of a SV40 fragment that restores the late leader region and includes the initiation codon for VP2. Both vectors were cloned as Bam Hl fragments in pBRM (a 2 kb fragment derived from pBR322 that contains the β-lactamase gene conferring resistance to ampicillin and the plasmid origin of replication).

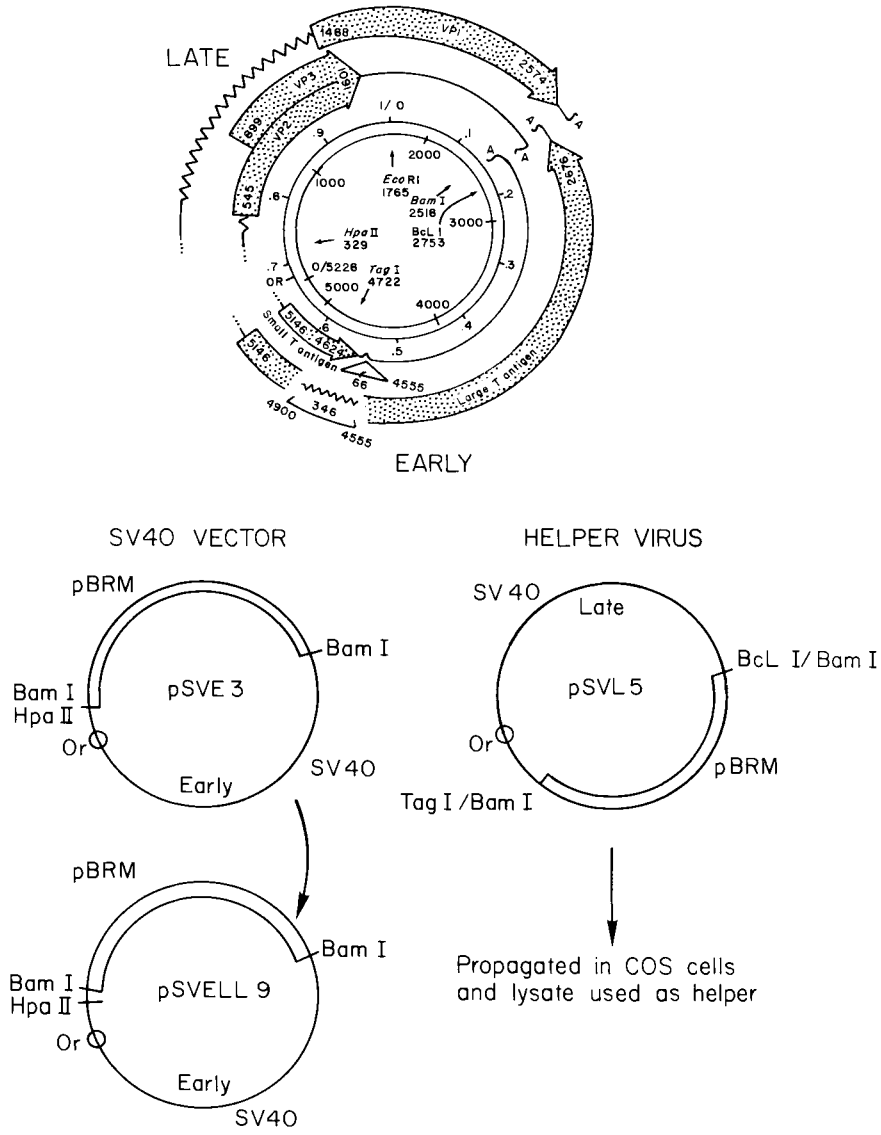

Figure 1: SV40 landmarks and schematic description of the SV-40 vectors and helper virus utilized to achieve expression of the influenza hemagglutinin (HA).

Cloning the HA Gene

The influenza A/WSN/33 HA gene was cloned as cDNA via the dG:dC tailing procedure into the Pst 1 site of pBR322 (1). Complete sequence analysis revealed that the gene contains a non-coding region of 32 nucleotides at the 5' end followed by coding region of 1695 nucleotides and a 3' non-coding region of 34 nucleotides (14). The HA clone (pHA 2-29) used for experiments reported here contains the entire structural gene including poly A addition site and lacks only the last 13 nucleotides at the 3' end of viral RNA which is not present in HA mRNA. The cloned HA fragment was further modified by treatment with nuclease Bal 31, Bam Hl linkers were added and it was recloned in the Bam Hl site of pBRM. This approach was undertaken in order to remove the dG:dC tails and the untranslated 5' region which might interfere with optimal expression when introduced into the SV40 vectors described above. Out of the 40 colonies that were analyzed, 13 had the correct insertion. Figure 2 shows 10 of these clones cleaved with Bam Hl.

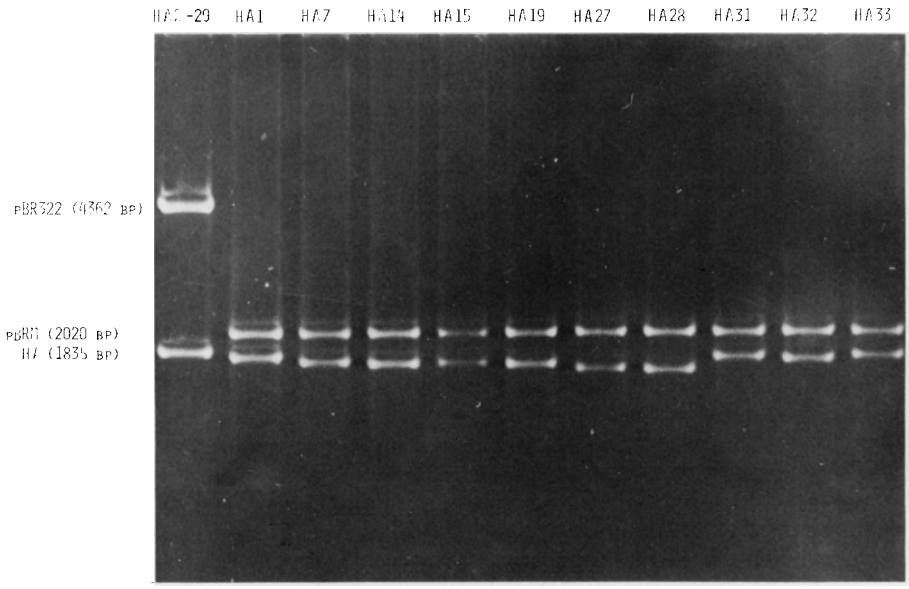

Figure 2: Bam Hl cleavage of the recloned HA gene. Plasmid preparations isolated from transformed E. coli cells were treated with Bam Hl, fractionated on 1% agarose gel and visualized under ultraviolet light by staining with ethidium bromide.

In order to select for those clones that contain the entire HA gene, end analysis was performed as follows: the isolated HA Bam Hl fragments were treated with calf intestinal alkaline phosphatase, labeled with [γ-^{32}P]ATP and cleaved with Taq I restriction endonuclease. For comparison, the original HA 2-29 clone was first cleaved with Taq I and only then end labeled with [γ-^{32}P]ATP. The fragments were analyzed on a 5% polyacrylamide gel as illustrated in Figure 3. It is noted that an average of 120 base pairs were removed from the original HA clone: about 50 and 70 from the 5' and 3', respectively.(Fig. 3B). DNA sequencing using the method of Maxam and Gilbert (15)

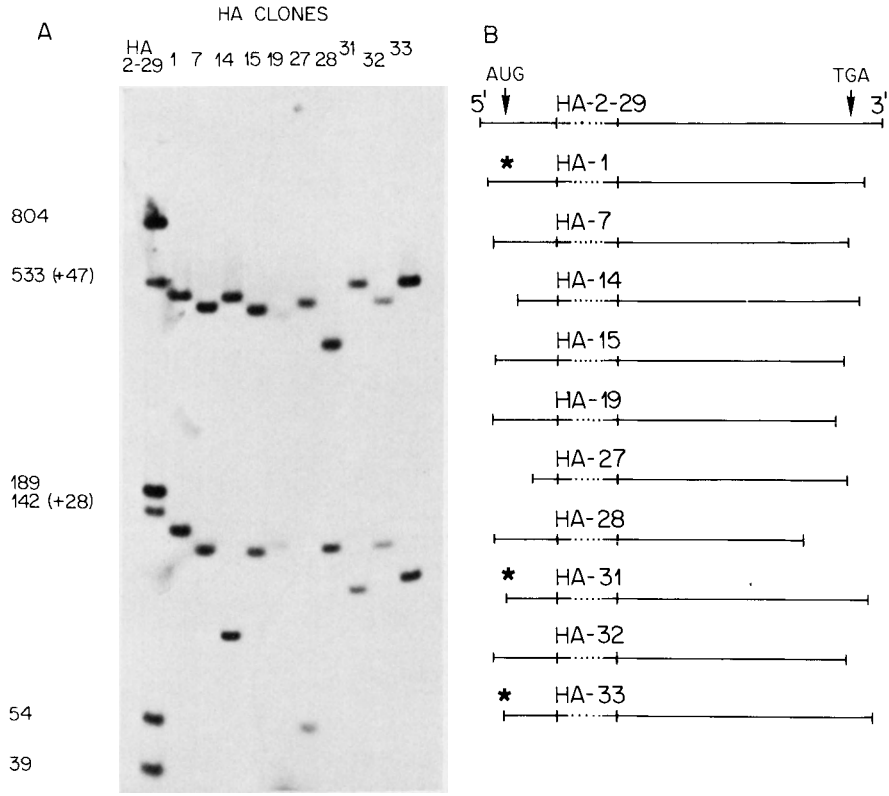

Figure 3: End analysis of the recloned HA gene. Plasmids bearing the HA gene were cleaved with Bam Hl and the HA fragment isolated, treated with alkaline phosphatase and the 5' end labeled with [γ-^{32}P]ATP. The HA fragment was then cleaved with Taq I and electrophoresed on 5% polyacrylamide gel. The original HA clone (2-29) was first restricted and only then end labeled.

was performed to analyze the termini of several shortened HA genes. This sequence data allowed us to identify HA genes which had both intact 5' and 3' coding regions as well as those in which deletions into one or the other end existed.

Three different HA clones (marked with asterisks, Fig. 3B) were selected for linkage to the SV40 vectors via complementary Bam H1 restriction termini . The structure of the SV40 recombinants carrying the HA insert in its two possible orientations is shown in Figure 4.

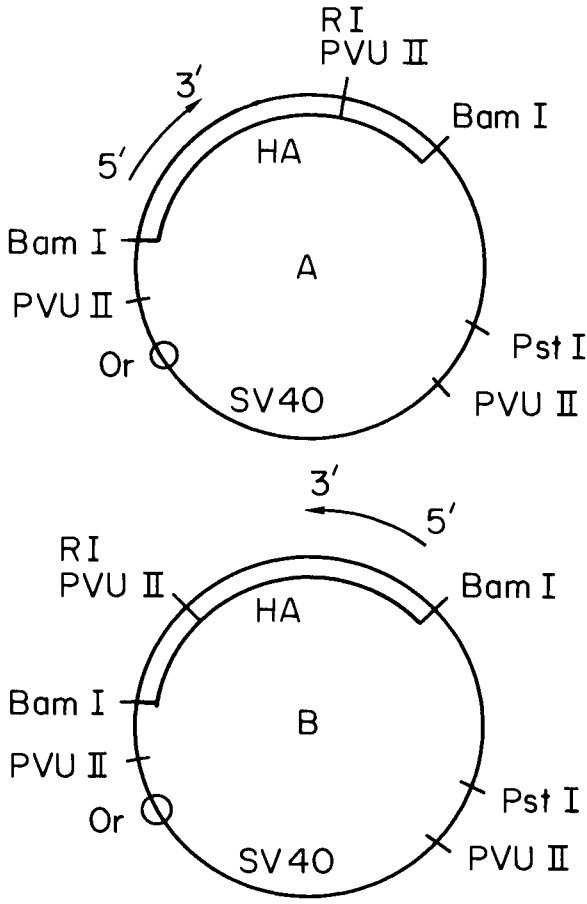

Figure 4: Possible arrangements of the SV40-HA recombinants.

Cloning in Monkey Cells

Since these recombinants contain intact early gene regions, and are about 5200 base pairs in size, they can be complemented for multiplication by an early mutant of SV40. We have used for his purpose SV40 early deletion mutants carrying a portion of pBR322 DNA in their early gene regions (Fig. 1). These mutants have been propagated in permissive monkey kidney cells transformed by specific origin deletion mutants of SV40 (16), thus generating helper virus stocks.

Secondary African green monkey kidney cells were transfected with the ligation products formed from the two SV40 vectors and the HA genes (free of pBRM). The ligation products were first separated on agarose gel and only those corresponding to unit length SV40 were purified and used for transfection. The ligation mixtures contained the following combinations: (a) HA1 x SVE3; (b) HA1 x SVELL9; (c) HA31 x SVELL9; and (d) HA33 x SVELL9. The recombinants were designated SV-HA 1 to 4, respectively. One to 2 days following the transfection, the cultures were infected with helper virus carrying completely functional late SV40 genes and thus able to complement the SV40-HA recombinants for multiplication. All virus stocks obtained after completion of the lytic infection (approximately 7 to 10 days after the DNA transfection) contained SV40-HA recombinants with the HA gene inserted with nearly equal frequency in either of the two possible orientations with respect to the SV40 late gene region (data not shown).

Expression of the Hemagglutinin in Monkey Cells

We have assayed for expression of the influenza HA protein in monkey cells infected with the SV40-HA recombinants using either immunoprecipitation of S^{35}-methionine labeled cell extracts (17) (Figure 5) or by indirect immunofluorescence (Figure 6). Using an antibody prepared in rabbits immunized with disrupted influenza virus, we readily detected production of HA-polypeptide. Interestingly, both SV40 vectors were capable of expressing the 70,000 dalton HA protein as judged by comigration in a 12.5% polyacrylamide gel of the immunoprecipitated S^{35}-labeled polypeptides with S^{35}-HA from extracts of cells infected with the original influenza virus strain (Figure 5). The reason why immunoprecipitated HA migrated as two separate bands is not entirely clear but may be due to a difference in glycosylation. Intracellular localization of HA antigen was studied by using indirect immunofluorescent assay. As shown in Figure 6 (panel C) HA antigen appears to be present in the cytoplasm of cells infected with SV-40 recombinants. We were unable to detect any HA production

by the SV-HA4 recombinant. Preliminary sequencing data suggest though that this HA clone is missing its initiator codon and that the gene is inserted in the wrong coding frame into the VP-2 locus.

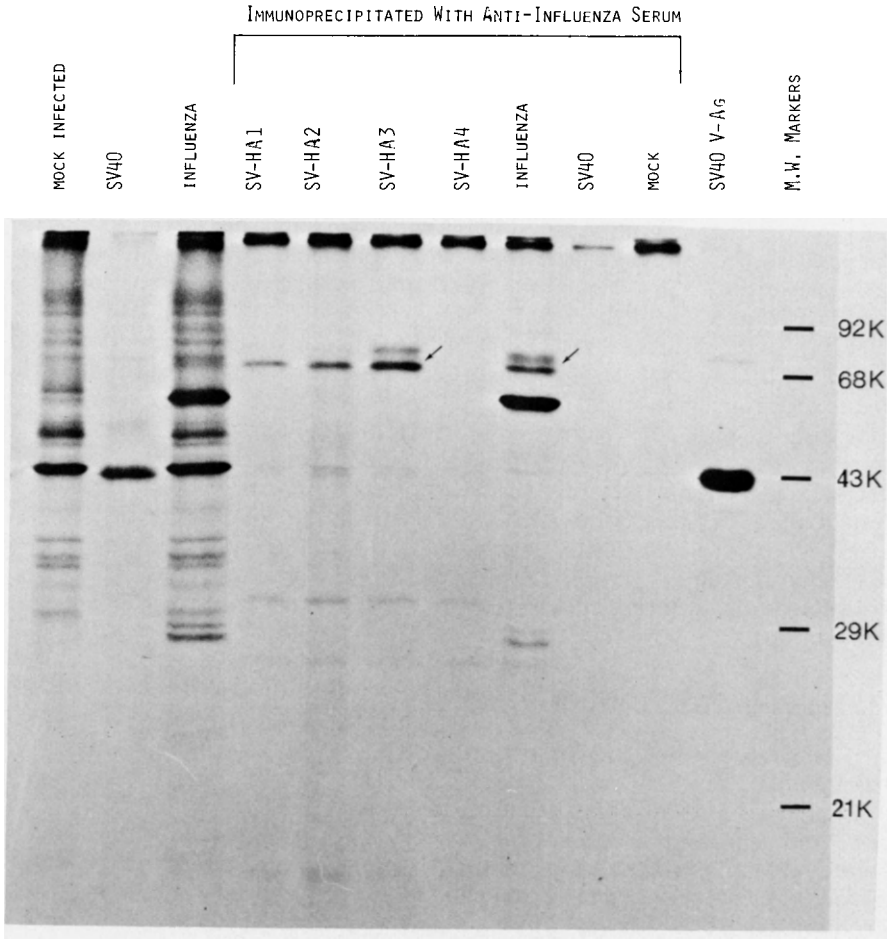

Figure 5: Detection of ^{35}S-HA by immunoprecipitation. Secondary AGMK cells were infected with the SV40 stocks indicated above each lane and labeled for 2 hr with ^{35}S-methionine at 45 hr postinfection in methionine-free medium. Cells infected with influenza virus were labeled at 15 hr postinfection. The extracts were immunoprecipitated with rabbit-anti influenza serum, fractionated on 12.5% SDS-polyacrylamide gel and the gel was then subjected to autoradiography for 7 days using an intensifying screen. Arrows indicate the location of the putative HA-0.

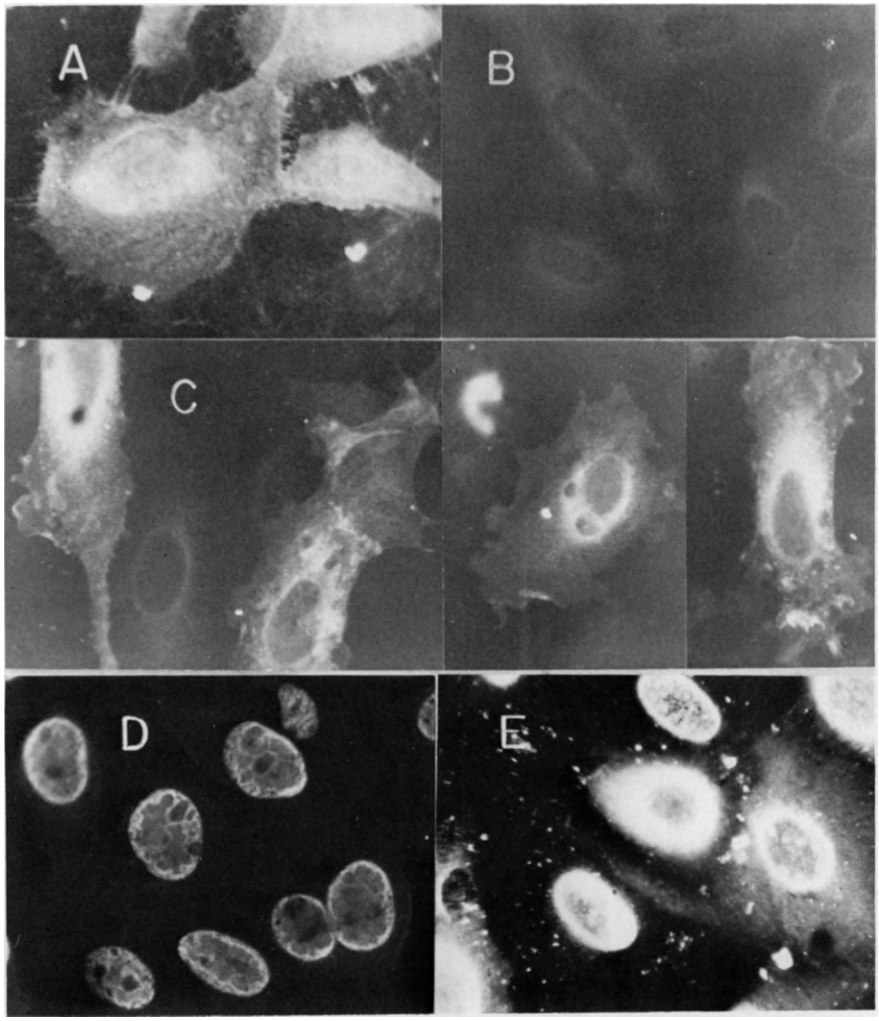

Figure 6: Detection of HA by immunofluorescence. Secondary AGMK cells were infected with influenza virus (panel A) with SV-HA4 (panel B) and SV-HA3 (panel C) and stained with anti-influenza serum. Panels D and E represent AGMK cells infected with SV-HA3 and stained with anti-SV40 T and V antiserum, respectively.

These results provide evidence for efficient expression of the influenza HA polypeptide; however, they indicate that the complete processing by proteolytic cleavage to yield HA1 and HA2 subunits does not take place since these smaller polypeptides are not detectable. Since this cleavage event occurs during influenza virus budding from infected cells, it is not surprising that it is not observed with these recombinants. The availability of SV40-HA recombinants should permit the examination of the biological effects of specific mutations introduced into the cloned HA gene and may be useful in complementation experiments with various temperature sensitive HA mutants of influenza. Finally, as additional RNA virus genes are cloned and their structure characterized in DNA prokaryotic vectors, their biological activities may be confirmed and manipulated through approaches analogous to these described in this paper.

ACKNOWLEDGEMENTS

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REFERENCES

1. Davis, A., Hiti, A. and Nayak, D.P. (1980). Gene 10,205.
2. Tooze, J., ed. (1980). DNA Tumor Viruses: Molecular Biology of Tumor Viruses (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), Part 2.
3. Mulligan, R.C. and Berg, P. (1980). Science 209,1422.
4. Gruss, P. and Khoury, G. (1981). Proc. Natl. Acad. Sci. U.S.A. 78,133.
5. Hamer, D.H. and Leder, P. (1979). Cell 18,1299.
6. White, D.O. (1974). Curr. Top. Microbiol. Immunol. 63,1.
7. Drzeniek, R., Seto, J.T. and Rott, R. (1966). Biochim. Biophys. Acta 128,547.
8. Laver, W.G. and Kilbourne, E.D. (1966) Virol. 30,493.
9. Hirst, G.K. (1942) J. Exp. Med. 75,49.
10. Klenk, H.D., Rott, R., Orlich, M. and Blödorn, J. (1975). Virol. 68,426.
11. Lazarowitz, S.G. and Choppin, P.W. (1975). Virol. 68,440.
12. Laver, W.G. and Webster, R.G. (1968). Virol. 34,193.
13. Laver, W.G. and Webster, R.G. (1972). Virol. 48,445.

14. Hiti, A., Davis, A. and Nayak, D.P. (1981). Virol. 110, in press.
15. Maxam, A.M. and Gilbert, W. (1977). Proc. Natl. Acad. Sci. U.S.A. 74,560.
16. Gluzman, Y. (1981). Cell 23,175.
17. Kessler, S.W. (1975). J. Immunol. 115,1617.

PROCESSING OF THE HEMAGGLUTININ

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SUMMARY

Processing of the hemagglutinin involves transport from the rough endoplasmic reticulum to the plasma membrane, glycosylation, and proteolytic cleavage. The tight coupling of these events is demonstrated in experiments in which mutants of fowl plague virus (FPV) with a temperature sensitive defect in transport have been analyzed. Two groups of such mutants have been characterized. With the first group (ts1, ts227), the hemagglutinin is arrested in the rough endoplasmic reticulum. With the second group (ts482, 532, 651), the hemagglutinin migrates to the Golgi apparatus, but does not reach the plasma membrane.

The distribution of the carbohydrate side chains on the FPV hemagglutinin has been elucidated. HA_1 has 4 type I side chains attached to asparagine residues 12, 28, 123, and 149. The potential attachment site at asparagine 231 is not glycosylated. HA_2 has a type II chain at asparagine 406 and a type I chain at asparagine 478. Comparison with other hemagglutinins demonstrates that the side chains in positions 12, 28, and 478 are conserved.

The cleavage site has been analyzed with H3, H7, and H10

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hemagglutinins. The available evidence indicates that proteolytic activation involves the action of a trypsin-like protease followed by the action of an exopeptidase of the carboxypeptidase B type: After *in vitro* cleavage with trypsin, which is paralleled by activation of infectivity, the N-terminus of HA_2 and presumably the C-terminus of HA_1 are identical to those obtained after *in vivo* cleavage. This indicates that the carboxypeptidase B activity is present in the virion. After cleavage with the non- activating enzymes thermolysin and chymotrypsin, the cleavage site is shifted by a few amino acids. These observations indicate that activation of infectivity requires a highly specific amino acid sequence at the cleavage site.

I. INTRODUCTION

The hemagglutinin of influenza virus is a glycoprotein of high importance for the biology of the virus and the nature of the disease. It is the major antigen at the surface of the virus and, due to its structural variability, it appears to be directly responsible for the uncontrolled recurrence of influenza epidemics in man. Another important aspect is the role which the hemagglutinin plays in initiation of infection. We know now that it has 2 essential functions in this process: (1) It is generally agreed that the hemagglutinin is responsible for adsorption of the virus to the cell surface. (2) There is increasing evidence for an involvement of the hemagglutinin in penetration by triggering fusion of the viral envelope with cellular membranes. Thus, cellular membranes exposed to influenza virus show fluidity changes similar to those observed after exposure to Sendai virus which is a well known fusing agent [Nicolau et al., 1978]. Exposure of cells resulted also in lysis by specifically sensitized cytotoxic T cells implying that the viral envelope has fused with the cell membrane [Kurrle et al., 1979]. Recently it could be shown that the hemagglutinin induces the fusion of liposomes with cell membranes [Huang et al., 1980a, b] and, under appropriate conditions cell fusion and hemolysis [Maeda and Ohnishi, 1980; Huang et al., 1981].

The biosynthesis of the hemagglutinin involves translation at membrane-bound ribosomes, insertion into the membrane of the rough endoplasmic reticulum, and transport to the plasma membrane [Klenk and Rott, 1980]. Insertion into the rough endo-

plasmic reticulum is mediated by a signal sequence at the amino terminus which is removed by cotranslational proteolytic cleavage [McCauley et al., 1980]. Another proteolytic event occurs at the posttranslational level where the precursor HA is cleaved into the fragments HA_1 and HA_2. Cleavage of HA is essential for the fusing capacity and, thus, for the infectivity of the virus. Cellular proteases are involved in this activation reaction, and, depending on the presence of an appropriate enzyme in a given cell, virus particles with cleaved or with uncleaved hemagglutinin may be formed [Klenk et al., 1975; Lazarowitz and Choppin, 1975]. On the other hand, there are also virus-specific variations in the susceptibility to cleavage. The hemagglutinin of some influenza A strains, such as FPV, is proteolytically activated in all host systems analyzed, whereas the hemagglutinin of other strains is activated only in a few hosts [Klenk et al., 1975]. Evidence has been presented that these differences account for variations in the pathogenicity of avian influenza viruses [Bosch et al., 1979].

In addition to proteolytic cleavage, processing of the hemagglutinin involves also covalent attachment of fatty acids presumably at the carboxy-terminal end, which may be necessary for the binding to the membrane [Schmidt and Schlesinger, 1980], and glycosylation. Glycosylation is initiated by the *en bloc* transfer of mannose-rich oligosaccharides from dolichol pyrophosphate to the polypeptides where they are attached by N-glycosidic linkages to asparagine residues [Schwarz et al., 1977; Nakamura and Compans, 1979; Keil et al., 1979]. This occurs in the rough endoplasmic reticulum. Upon arrival of the hemagglutinin in the Golgi apparatus some of the side chains undergo extensive modifications involving removal of mannose and attachment of galactose and fucose residues. Thus, 2 major types of carbohydrate side chains are found on the glycoproteins: The mannose-rich type II containing only mannose and glucosamine and the complex type I that contains in addition galactose and fucose [Schwarz et al., 1977; Nakamura and Compans, 1978; Collins and Knight, 1978; Ward and Dopheide, 1980].

Transport, glycosylation, and proteolytic cleavage are tightly coupled events as has been demonstrated by analysis of a ts mutant where the hemagglutinin was arrested at the rough endoplasmic reticulum [Lohmeyer and Klenk, 1979]. We present here other mutants with defects in the transport of the hemagglutinin. Furthermore, we will show the exact arrangement of the carbohydrate side chains on the FPV hemagglutinin. Finally

this contribution will be concerned with the enzymes responsible for cleavage of HA and with the structure of the cleavage site.

II. MATERIALS AND METHODS

Viruses. Influenza strain A/Port Chalmers/1/73 (H3N2) (recombinant MRC11), A/FPV/Rostock/34 (H7N1), A/FPV/Dutch/27 (H7N7), A/Chick/Germany/N/49 (H1ON7), and the FPV mutants ts 227 [Scholtissek and Bowles, 1975], ts1, ts482, ts532, and ts651 [I. Koennecke, thesis, Giessen, 1981] have been used. Virus growth in eggs or in chick embryo fibroblasts, labeling with radioactive isotopes, virus purification, and isolation of the hemagglutinin by preparative polyacrylamide gel electrophoresis were done as described elsewhere [Schwarz et al., 1977]. The procedure used for 2-dimensional isoelectric focusing and electrophoresis has been described elsewhere [Kohama et al., 1981].

Carbohydrate analysis. Glycopeptides were prepared by Pronase digestion and isolated by chromatography on Biogel P6 columns [Keil et al., 1979]. Type II side chains were also obtained by cleavage with endoglycosidase H. Constituent sugars were analyzed by permethylation followed by gas liquid chromatography combined with mass spectroscopy. Localization of the side chains on the FPV hemagglutinin is described in the text.

Analysis of amino acid sequences. Amino-terminal sequences were determined by a microscale modification of the dansyl-Edman degradation of unlabeled polypeptides [Weiner et al., 1972] and by Edman degradation of polypeptides that were specifically labeled with radioactive amino acids. Carboxy termini were determined (1) by digestion with carboxypeptidase A followed by amino acid analysis of the released residues and (2) by electrophoretic analysis of the carboxy-terminal cyanogen bromide fragment that had been specifically labeled with radioactive amino acids.

III. RESULTS AND DISCUSSION

A. Mutants of Fowl Plague Virus with a Temperature Sensitive Defect in the Transport of the Hemagglutinin

The ts mutant of FPV analyzed in the previous study was ts227 [Lohmeyer and Klenk, 1979]. We now report on mutants ts1, ts482, ts532, and ts651 which all have ts defects in the synthesis of the hemagglutinin. When growth curves were analyzed at permissive (33°) and at non-permissive temperature (41°), the mutants could be subdivided into 2 different groups. A member of the first group is ts1 where, at the non-permissive temperature, biologically active hemagglutinin can be detected neither in the medium nor in the cell (Fig. 1). The only other mutant that falls into this group is the previously analyzed ts227. The second group (ts482, ts532, ts651) can be discriminated from the first one by different growth curves. As demonstrated on the example of ts651 hemagglutinating activity is produced at 41° in the cell, but is not released into the medium (Fig. 1).

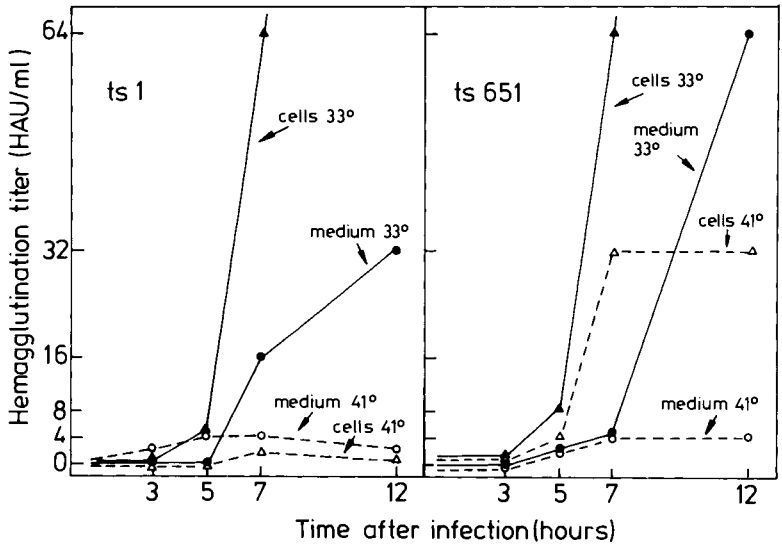


Figure 1: Growth curves of FPV mutants ts1 and ts651. Cell-bound and released hemagglutinin was measured. Cells were disrupted by 3 cycles of freezing and thawing.

When infected cells are analyzed by polyacrylamide gel electrophoresis it becomes clear that the hemagglutinin protein is synthesized at the non-permissive temperature with both groups (Fig. 2). However, there are distinct differences between the groups in the extent of hemagglutinin processing. With ts1 the hemagglutinin is present at 41° predominantly in the uncleaved form. It can be labeled with glucosamine (Fig.2) and mannose, but not with galactose and fucose (data not shown). Thus, it appears that the ts1 hemagglutinin is arrested at the non-permissive temperature in the rough endoplasmic reticulum, as is the case with the ts227 hemagglutinin [Lohmeyer and Klenk, 1979]. The hemagglutinin of ts651, however, is readily cleaved under these conditions (Fig. 2).

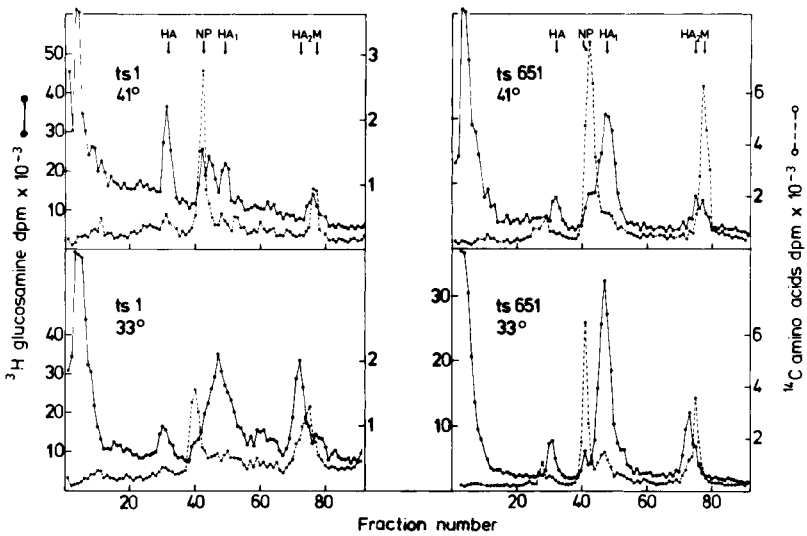


Figure 2: Protein synthesis in chick embryo cells infected with FPV mutants ts1 and ts651. Experiments were carried out at permissive (33°) and non-permissive (41°) temperatures. 4 Hours after infection cells were labeled for 3 hours with 6-[^{3}H]-glucosamine and a mixture of [^{14}C]-amino acids. Polyacrylamide gel electrophoresis was carried out on cylindrical gels.

It can be labeled with glucosamine (Fig. 2) as well as with mannose, galactose, and fucose (data not shown). Similar results have been obtained, when ts482 and ts532 were analyzed.

These observations indicate that the hemagglutinin of these mutants reaches the Golgi apparatus at 41°, and that therefore proteolytic cleavage and remodelling of the carbohydrate side chains can occur. To find out whether the transport is terminated in this organelle or whether it proceeds to the plasma membrane, we have looked for the hemadsorbing capacity of cells that had been infected with the mutants for 7 hours. With all mutants hemadsorption was observed only at permissive, but not at non-permissive temperature.

The data show that with all mutants virus particles are not produced at non-permissive temperature because the transport of the hemagglutinin to the cell surface is blocked. The mutants fall into two groups with respect to the organelle in which the transport is blocked. The hemagglutinin of ts1 and ts227 is arrested already in the rough endoplasmic reticulum, whereas with ts482, ts532, and ts651 transport of the hemagglutinin is terminated in the Golgi apparatus. These observations suggest that different structural properties of the hemagglutinin are involved in the transport from the rough endoplasmic reticulum to the Golgi and from the Golgi to the plasma membrane.

B. Glycosylation

1. The carbohydrate side chains of the fowl plague virus hemagglutinin. Based on data from glycopeptide analyses and from glycosylation inhibition studies we have previously estimated that the FPV hemagglutinin contains 5 - 6 oligosaccharide side chains and that 2 of these are located on HA_2 [Schwarz et al., 1977]. The elucidation of the amino acid sequence revealed that the polypeptide has a total of 7 potential carbohydrate attachment sites [Porter et al., 1979]. For localizing the side chains we followed a procedure schematically described in Fig. 3. Fragments of the hemagglutinin were prepared by treatment with cyanogen bromide, V8 protease from Staphylococcus aureus, and trypsin. The position of the fragments was determined by radioactive labeling of the sugars and of specific amino acids. For instance, 2 cyanogen bromide fragments containing carbohydrate attachment sites are obtained from HA_2. One of these can be labeled by both tryptophan and tyrosine, whereas the other one contains only tyrosine. The results of this study are summarized in Fig. 4. HA_1 has 4 side chains which are all of type I. One

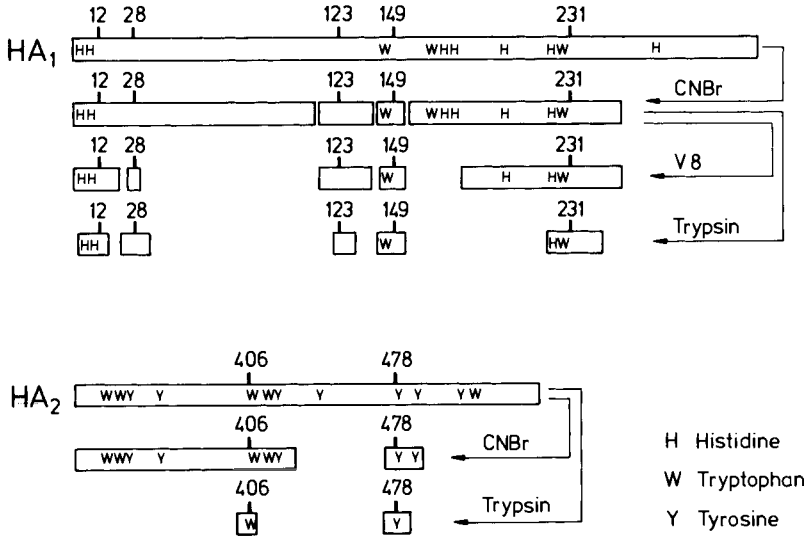


Figure 3: Fragmentation of the FPV hemagglutinin used for identification of the carbohydrate side chains. Cyanogen bromide fragments were isolated by polyacrylamide gel electrophoresis. Tryptic fragments were isolated by chromatography on Biogel P6 and on AG50x2 columns. Digests of Staphylococcus aureus V8 protease were analyzed by polyacrylamide gel electrophoresis. The positions of all potentiall attachment sites and of some of the amino acids used in radioactive labeling are indicated.

potential attachment site (asparagine 231) is not glycosylated. Both attachment sites of HA_2 are glycosylated. The only type II side chain of the hemagglutinin is attached to asparagine 406, whereas asparagine 478 has a type I side chain.

The structure of the type II side chain has been analyzed in some detail. This chain exhibits considerable heterogeneity as demonstrated by the observation that 4 different oligosaccharides (HM 1-4) can be discriminated after digestion with endoglycosidase H (Fig. 5).

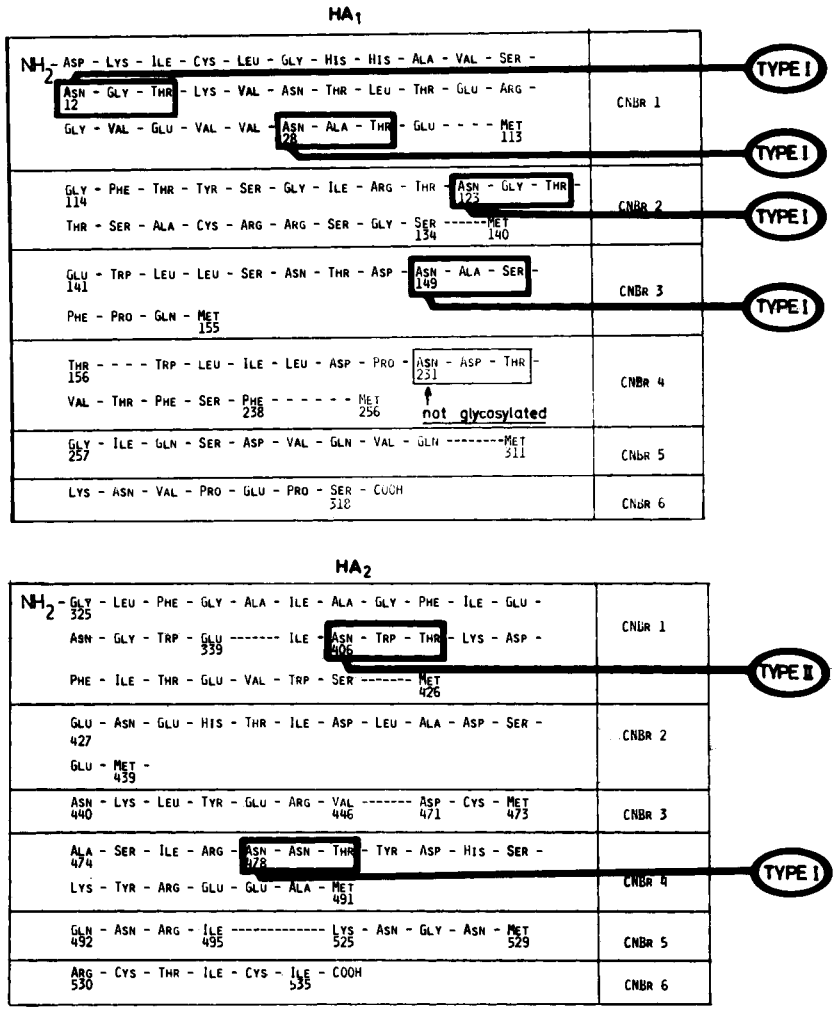


Figure 4: Position of the oligosaccharides on the hemagglutinin of strain A/FPV/Rostock/34. The amino acid sequences [Porter et al., 1979] at the amino-terminal and carboxy-terminal ends of the cyanogen bromide fragments and at the glycosylation sites are indicated.

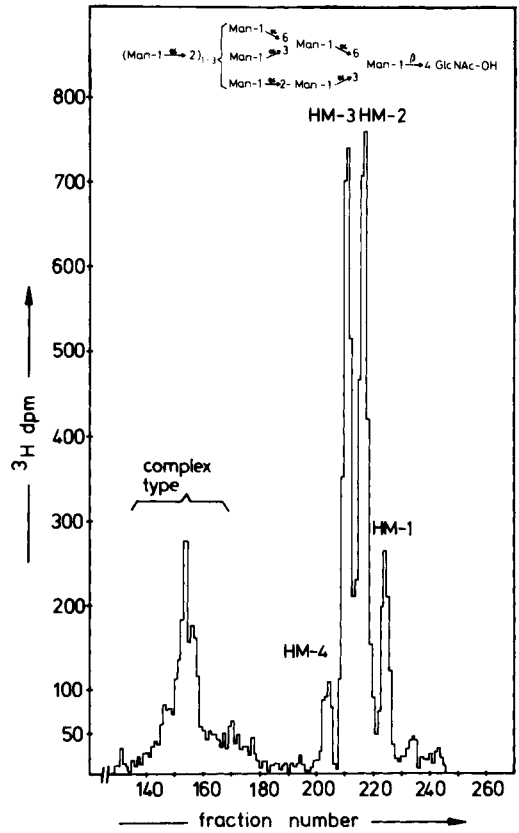


Figure 5: Structural heterogeneity of the type II oligosaccharide of the FPV hemagglutinin. Pronase digests of HA_2 were subjected to endoglycosidase H treatment. The oligosaccharides derived from the type II side chain (HM 1-4) were separated from type I glycopeptides (not susceptible to endoglycosidase H) by chromatography on a Biogel P4 column. The common core structure of oligosaccharides HM 2, 3, and 4 is also shown.

Analysis of the permethylated oligosaccharides in conjunction with studies employing endoglycosidase D has revealed that HM2, HM3, and HM4 possess 7, 8, and 9 mannose residues, respectively, and the common core structure shown in Fig. 5. It is interesting to note that this heterogeneity appears to be quite stable, since exactly the same oligosaccharide pattern has been obtained from several different virus preparations.

Our data indicate that, at a given glycosylation site, only one type of side chain is present. Thus, the type II side chain of the FPV hemagglutinin is always attached to asparagine 406. Type I is never observed in this position. There is evidence that the side chains of type I located in different positions on the polypeptide are not identical as indicated by differences in sugar composition and sulfation [Ward and Dopheide, 1980; Nakamura et al., 1980; W. Keil, manuscript in preparation]. Thus, each glycosylation site has a specific carbohydrate side chain. However, Fig. 5 demonstrates that a specific side chain may exhibit considerable microheterogeneity. Structural analysis of the hemagglutinin oligosaccharides is therefore a rather complicated project.

2. Strain-specific variations in the glycosylation pattern. Although the complete amino acid sequence of the hemagglutinin and, thus, the position of the potential carbohydrate attachment sites have been elucidated with a considerable number of influenza A strains, there are only 3 hemagglutinins where information is available on the nature of the carbohydrate attached to a specific glycosylation site. Besides the FPV hemagglutinin, these are the hemagglutinins of strains A/Japan/305/57 (H2) and A/Memphis/102/72 (H3). Fig. 6 demonstrates that some attachment sites appear to be conserved (e. g. those around sequence positions 40, 50, and 510), whereas others show a high degree of variability. It is interesting to note that in the three-dimensional model of the hemagglutinin [Wilson et al., 1981] the conserved side chains, even though on opposite ends on the unfolded polypeptide, are located in close neighbourhood to each other at the base of the spike. This may indicate that these side chains have a special function in maintaining the structure of the hemagglutinin. The diversity in the oligosaccharide patterns of the hemagglutinins of different influenza A strains all grown in the same host strengthens the concept that the primary structure of the polypeptide is an important determinant for the carbohydrate moiety of a glycoprotein. However, beyond that virtually nothing is known about the rules according to which a specific carbohydrate side chain is attached to a given glycosylation site. The wide variability in the polypeptide as well as in the carbohydrate portion makes the influenza virus hemagglutinin a unique system to study this problem.

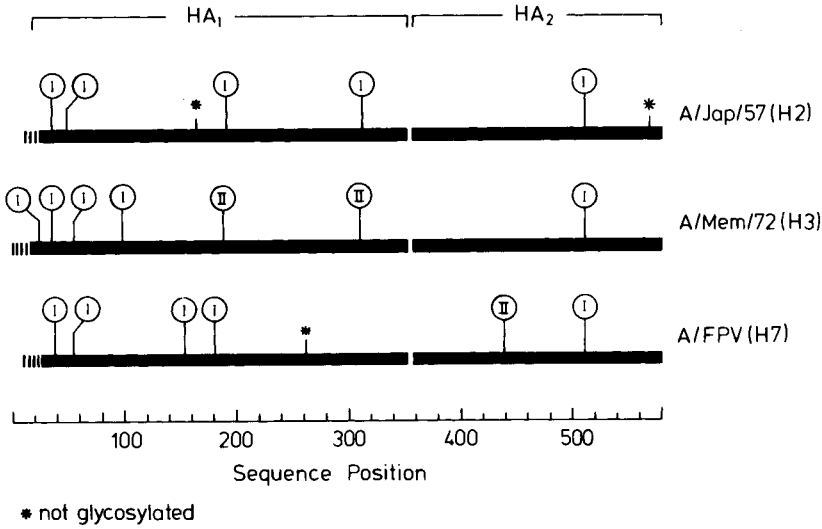


Figure 6: Arrangement of carbohydrate side chains on 3 different hemagglutinins. The data of the H2 hemagglutinin are from Waterfield and coworkers [1980], Klenk and coworkers [1980], and Brown and coworkers [1981]. The data on the H3 hemagglutinin are from Ward and Dopheide [1980]. Cysteine residues have been used for the alignment of the polypeptide chains.

C. The Mechanism of Proteolytic Activation

1. The cleavage sites of H3 and H10 hemagglutinins. Proteases of different specificities are able to cleave HA, but activation is observed only after cleavage with trypsin or trypsin-like enzymes [Lazarowitz and Choppin, 1975; Klenk et al., 1977]. These observations suggested that cleavage of a

specific peptide bond is required for activation. It was also of interest to find out if the same peptide bonds are cleaved by in vivo activation and by in vitro activation with trypsin. Therefore, comparative sequence analyses were carried out on hemagglutinin that has been cleaved either in vivo or in vitro using proteases of various specificities.

The results obtained from hemagglutinin of serotype H3 are shown in Table I.

TABLE I The cleavage site of H3 hemagglutinins

Uncleaved HA [a]	-GLU-LYS-GLN-THR-ARG-GLY-LEU-PHE-GLY-ALA-
Cleavage in vivo [b]	-GLU-LYS-GLN-THR ARG GLY-LEU-PHE-GLY-ALA-
Cleavage by trypsin [c]	-GLU-LYS-GLN-THR ARG GLY-LEU-PHE-GLY-ALA-
Cleavage by thermolysin [c]	-GLU-LYS-GLN-THR-ARG-GLY LEU-PHE-GLY-ALA-

C-Terminus of HA_1 ▒▒▒ Sequences identified by amino acid analysis ———
N-Terminus of HA_2 ▓▓▓

a) Strain A/Memphis/102/72 [Sleigh et al., 1980]
b) Strain A/Memphis/102/72 [Ward and Dopheide, 1980] and A/Port Chalmers/1/73 [Laver et al., 1980]
c) Strain A/Port Chalmers/1/73

The sequence analysis of the precursor HA [Sleigh et al., 1980; Min-Jou et al., 1980] and of the cleavage product [Ward and Dopheide, 1980] had already shown that an arginine residue is eliminated at the cleavage site, when cleavage takes place in vivo, and that threonine is at the carboxy terminus of HA_1 and glycine at the amino terminus of HA_2. Here we show that the same amino terminus is found after cleavage with trypsin, whereas cleavage with the non-activating enzyme thermolysine shifts the amino terminus to the adjacent leucine. With the H3 hemagglutinin amino acid sequences have not been directly analyzed yet at the carboxy terminus of HA_1 after in vitro cleavage with these enzymes. Pertinent information has been obtained, however, by the following approach. Based on the assumption that elimination of an arginine residue should result in a shift of the hemagglutinin towards a more acidic isoelectric point, we have performed isoelectric focussing studies. Such a shift can be indeed observed, not only when the hemagglutinin is cleaved in vivo (data not shown), but also after in vitro cleavage with trypsin (Fig. 7). However, after cleavage by thermolysin the shift is not observed (Fig.7). Thus, it appears that arginine is eliminated after trypsin, but not after thermolysin treatment.

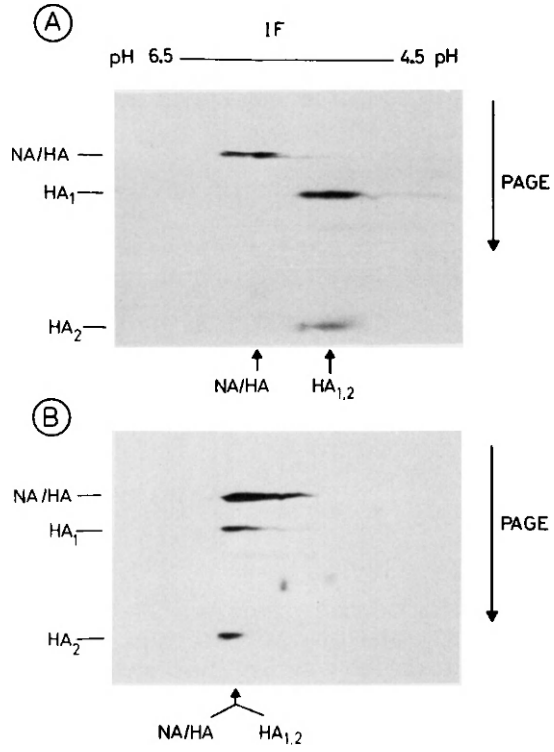


Figure 7: Two-dimensional isoelectric focusing and electrophoresis of the glycoproteins of strains A/Port Chalmers/1/73 (H3N2). Virions labeled with [^{3}H]-amino acids and grown in chick embryo fibroblasts have been used. The hemagglutinin was cleaved by in vitro treatment with trypsin (A) and thermolysin (B). Both samples were mixed with virus containing the uncleaved hemagglutinin. After disintegration in 2 % NP40 and 9 M urea at 25°, viral glycoproteins were analyzed on polyacrylamide gels by electrofocusing under non-reducing conditions in horizontal direction followed by electrophoresis under reducing conditions in vertical direction (Kohama et al., 1981]. The origin is in the upper right corner. Identical results were obtained, when virus was pretreated with 1.5 % SDS.

These observations have been further substantiated by similar studies performed on strain A/Chick/Germany/49 (H1ON7). As shown in Table II, again identical amino termini of HA_1 can be observed after in vivo cleavage and after cleavage with trypsin or the trypsin-like enzyme acrosin. We have obtained tentative evidence that the carboxy termini of HA_1 are also identical, and that arginine is removed under these conditions. On hemagglutinin cleaved by the non-activating enzymes thermolysin and chymotrypsin, sequence analyses have been performed at the amino terminus of HA_2 as well as on the carboxy terminus of HA_1. The results demonstrate clearly that single peptide bonds are cleaved under these conditions, and that the cleavage site is shifted by one or three amino acids in carboxy-terminal direction. We have also analyzed the H10 hemagglutinin by isoelectric focusing. Again, there was a shift towards a more acidic isoelectric point after in vivo cleavage or cleavage by trypsin, whereas thermolysin and chymotrypsin cleavage had no effects (data not shown).

TABLE II The cleavage site of the hemagglutinin of strains A/Chick/Germany/49 (H1ON7)

Uncleaved HA	- X - ARG-GLY-LEU-PHE-GLY-ALA-ILE-ALA-GLY-PHE-	inactive
Cleavage in vivo	- X* ARG GLY-LEU-PHE-GLY-ALA-ILE-ALA-GLY-PHE-	active
Cleavage by trypsin	- X* ARG GLY-LEU-PHE-GLY-ALA-ILE-ALA-GLY-PHE-	active
Cleavage by acrosin	- X ARG GLY-LEU-PHE-GLY-ALA-ILE-ALA-GLY-PHE-	active
Cleavage by thermolysin	- X - ARG-GLY LEU-PHE-GLY-ALA-ILE-ALA-GLY-PHE-	inactive
Cleavage by chymotrypsin	- X - ARG-GLY-LEU-PHE GLY-ALA-ILE-ALA-GLY-PHE-	inactive

C-Terminus of HA_1 ▒▒ Identified sequences ———

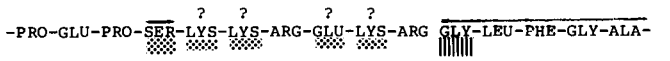
N-Terminus of HA_2 ||| Identical, not identified amino acids X*

The observations made on the cleavage site of the H3 and the H10 hemagglutinin allow the following conclusions. Since trypsin can cleave only the arginine-glycine bond, but not (in the case of the H3 hemagglutinin) the threonine-arginine bond, it appears that after the initial action of trypsin or a trypsin-like endoprotease, a proteolytic enzyme of different specificity, perhaps an exopeptidase of the carboxy peptidase B type, is involved in the cleavage reaction as has been suggested before by us and others [Ward and Dopheide, 1978; Klenk et al., 1980]. The observation presented here that argi-

nine is eliminated, when the hemagglutinin is cleaved in vitro with trypsin added as the only enzyme to purified virus, indicates that the carboxypeptidase is present in the virion and that it may be a virus-specific enzymatic activity. The observation that arginine is not eliminated when glycine or glycine-leucine-phenylalanine are present at its carboxy-terminal end as is the case after thermolysin or chymotrypsin cleavage, further supports the involvement of a carboxypeptidase B-like activity, since this enzyme cleaves only arginine and lysine in carboxy-terminal position. The results obtained with chymotrypsin and thermolysin demonstrate also that a shift of the cleavage site by only three or even a single amino acid is enough to yield inactive hemagglutinin. This observation is compatible with the studies of Richardson and coworkers [1980] who, by a different approach, found that activation of infectivity requires a specific sequence at the amino terminus of HA_2.

2. The cleavage site of the FPV hemagglutinin. The cleavage site of the FPV hemagglutinin (H7) is shown in Table III. The amino terminus of HA_2 is glycine as is the case with all other hemagglutinins. The carboxy terminus of HA_1, however, appears to be heterogenous. We have previously reported that serine is located in this position as indicated by carboxypeptidase A treatment [Klenk, 1980]. However, analysis of the carboxy-terminal cyanogen bromide fragments of HA_1 showed heterogeneity suggesting that HA_1 may terminate in several other positions as tentatively indicated in Table III. Despite of these uncertainties the cleavage site of the FPV hemagglutinin clearly differs from those of all other hemagglutinins sequenced to date by an intervening sequence of several basic amino acids instead of just a single arginine residue. As has already been pointed out above the FPV hemagglutinin differs also from most other hemagglutinins in its high susceptibility to proteolytic cleavage. It is therefore conceivable that the differences in the cleavage site account for the differences in proteolytic activation and, thus, in host range and in pathogenicity. This concept is supported by a comparative study of a series of pathogenic and apathogenic avian influenza A strains all belonging to serotype H7. It has been found that the acidic shift in the isoelectric point that parallels cleavage of the hemagglutinin is wider with the pathogenic than with the apathogenic strains. This observation suggests that a relatively large intervening sequence containing several basic amino acids is a common feature of the pathogenic strains.

TABLE III The cleavage site of the hemagglutinin of strain A/FPV/Rostock/34 (H7N1)



C-Terminus of HA_1 N-Terminus of HA_2

Sequences identified by amino acid analysis

The sequence of the uncleaved HA is from Porter et al. [1979].

ACKNOWLEDGMENTS

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REFERENCES

Bosch, F. X., Orlich, M., Klenk, H.-D., and Rott, R. (1979). Virology 95, 197.
Brown, L. E., Ward, C. W., and Jackson, D. C. (1981). J. Immunol., in press.
Collins, J. K., and Knight, C. A. (1978). J. Virol. 26, 457.
Huang, R.T.C., Wahn, K., Klenk, H.-D., and Rott, R. (1980a). Virology 104, 294.
Huang, R.T.C., Rott, R., Wahn, K., Klenk, H.-D., and Kohama, T. (1980b). Virology 107, 313.
Huang, R.T.C., Rott, R., and Klenk, H.-D. (1981). Virology, in press.

Keil, W., Klenk, H.-D., and Schwarz, R. T. (1979). J. Virol. 31, 253.
Klenk, H.-D., Rott, R., Orlich, M., and Blödorn, J. (1975). Virology 68, 426.
Klenk, H.-D., Rott, R., and Orlich, M. (1977). J. Gen. Virol. 36, 151.
Klenk, H.-D., and Rott, R. (1980). Current Topics Microbiol. Immunol. 90, 19.
Klenk, H.-D., Garten, W., Keil, W., Niemann, H., Schwarz, R. T., and Rott, R. (1980). In "Biosynthesis, Modification, and Processing of Cellular and Viral Polyproteins" (G. Koch and D. Richter, eds.), p. 175. Academic Press, New York.
Klenk, H.-D. (1980). In "Structure and Variation in Influenza Virus" (W. G. Laver, and G. Air, eds.) p. 123. Elsevier-North Holland.
Kohama, T., Garten, W., and Klenk, H.-D. (1981). Virology, in press.
Laver, W. G., Air, G. M., Dopheide, T. A., and Ward, C. W. (1980). Nature 283, 454.
Lohmeyer, J., and Klenk, H.-D. (1979). Virology 93, 134.
Maeda, T., and Ohnishi, S. (1980). FEBS Letters 122, 283.
McCauley, J., Skehel, J., Elder, K., Gething, M.-J., Smith, A., and Waterfield, M. (1980). In "Structure and Variation in Influenza Virus" (W. G. Laver, G. Air, eds.), p. 97, Elsevier-North Holland.
Min Jou, W., Verhoeyen, M., Devos, R., Saman, E., Huylebroeck, D., van Rompuy, L., Fang, R. X., and Fiers, W. (1980). In "Structure and Variation in Influenza Virus" (W. G. Laver, G. Air, eds.), p. 63. Elsevier-North Holland.
Nakamura, K., and Compans, R. W. (1978). Virology 86, 432.
Nakamura, K., and Compans, R. W. (1979). Virology 93, 31.
Nakamura, K., Brown, A. S., and Compans, R. W. (1980). Virology 107, 208.
Kurrle, R., Wagner, H., Röllinghoff, M., and Rott, R. (1979). Eur. J. Immunol. 9, 107.
Lazarowitz, S. G., and Choppin, P. W. (1975). Virology 48, 440.
Nicolau, C., Klenk, H.-D., Reimann, A., Hildenbrand, K., and Bauer, H. (1978). Biochim. Biophys. Acta 511, 83.
Porter, A. G., Barber, C., Carey, N. H., Hallewell, R. A., Threfall, G., and Emtage, J. S.(1979). Nature 282, 471.
Richardson, C. D., Scheid, A., and Choppin, P. W. (1980). Virology 105, 205.
Rott, R., Reinacher, M., Orlich, M., and Klenk, H.-D. (1980). Arch. Virology 65, 123.
Schmidt, M. F. G., and Schlesinger, M. J. (1980). Zbl. Bakt. Hyg. I. Abt. Orig. A248, 17.
Scholtissek, C., and Bowles, A. L. (1975). Virology 67, 576.

Schwarz, R. T., Schmidt, M. F. G., Anwer, U., and Klenk, H.-D. (1977). J. Virol. 23, 217.

Sleigh, M. J., Both, G. W., Brownlee, G. G., Bender, V. J., and Moss, B. A. (1980). In "Structure and Variation in Influenza Virus" (W. G. Laver, G. Air, eds.), p. 69. Elsevier-North Holland.

Ward, C., and Dopheide, T. (1980). In "Structure and Variations in Influenza Virus" (W.G. Laver, G. Air, eds.). p. 27. Elsevier-North Holland.

Waterfield, M., Gething, M.-J., Scrace, G., and Skehel, J. (1980). In "Structure and Variations in Influenza Virus" (W. G. Laver, G. Air, eds.). p. 11. Elsevier-North Holland.

Weiner, A. M., Platt, T., and Weber, K. J. (1972). J. Biol. Chem. 247, 3242.

Wilson, I. A., Skehel, J. J., and Wiley, D. C. (1981). Nature 289, 366.

DIRECTIONAL TRANSPORT OF VIRAL GLYCOPROTEINS IN POLARIZED EPITHELIAL CELLS[1]

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ABSTRACT

In the MDCK line of canine kidney cells, influenza virus assembly has been observed to occur exclusively at the apical surface, whereas formation of vesicular stomatitis virus (VSV) occurs only at basolateral membranes, beneath tight junctions. These junctional complexes prevent antiviral antibody from penetrating to the site of VSV maturation; as a consequence, VSV plaque formation in MDCK cells is not prevented by including neutralizing antibody in the agar overlay.

Despite the restriction of influenza and VSV assembly sites to distinct membrane domains, MDCK cells doubly infected with both viruses yield a high proportion of VSV particles containing influenza viral glycoproteins in their envelopes. However, the formation of such particles lags behind that of wild type VSV, and occurs predominantly after the first detectable cytopathic effects in the monolayer. Examination of thin section of doubly infected cells revealed that polarity of maturation sites was maintained for both viruses until later times post-infection, when destruction of junctional complexes was evident. These results indicate that directional transport of viral glycoproteins to distinct membrane domains continues in doubly infected MDCK cells. At later times post infection, cytopathic effects lead to disappearance of the junctional complexes which separate the apical and basolateral membrane

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domains, probably enabling lateral diffusion of glycoprotein in the plane of the membrane, and subsequent formation of phenotypically mixed particles.

Ionophores specific for monovalent cations, such as monensin, have been reported to inhibit the secretion of secretory glycoproteins and the membrane appearance of cell surface glycoproteins by preventing their exit from the Golgi complex. We observed that the replication of VSV in MDCK cells was drastically inhibited by monensin, with a block occurring in the transport of the G protein to the cell surface. In contrast, the replication of influenza virus was not affected by high concentrations of the ionophore. VSV components in monensin-treated cells appeared to accumulate in dilated cytoplasmic vacuoles, whereas influenza virus-infected cells contained dilated vacuoles that appeared empty. These observations indicate that two distinct systems exist for transport of membrane glycoproteins to MDCK cell surfaces; the pathway utilized by VSV is monensin-sensitive, and that utilized by influenza virus is monensin-resistant.

INTRODUCTION

Cells of the Madin Darby canine kidney (MDCK) line exhibit many of the features of normal secretory epithelia, including the presence of tight junctions between adjacent cells, a spontaneous electrical potential between the upper and lower cell surfaces, and a vectorial transport of fluid from the apical to basal surface (1-5). When injected into baby nude mice, these cells form epithelial sheets lining fluid filled glands with tight junctions between cells, and with apical cell surfaces oriented toward the central lumen (5). Recently it has been reported that the assembly of VSV occurs exclusively at the basolateral membranes in confluent MDCK cell monolayers, whereas influenza and several parainfluenza viruses bud only from the free apical surface (6). We have previously investigated the possible role of differences in glycosylation of viral glycoproteins in determining the maturation sites of enveloped viruses in MDCK cells (7). Using tunicamycin, an inhibitor of formation of asparagine-linked oligosaccharides (8), we demonstrated that the maturation sites of VSV and influenza virus were not altered by complete inhibition of glycosylation. However, it is likely that the site of insertion of viral glycoproteins determines the viral maturation site, since glycoproteins of VSV and influenza virus were found localized in the same membrane domain where virus maturation occurred (9). Thus, it is likely that structural features of the polypeptide backbone are involved in directing viral glycoproteins to specific membrane domains. In the present paper, we report the results of studies designed to further characterize the cellular pathways involved in

transport of viral glycoproteins to distinct membrane domains in epithelial cell monolayers.

MATERIALS AND METHODS

The methods employed in the present studies are described in detail elsewhere (7,10,12).

RESULTS

Antibody-resistant VSV Plaque Formation in Epithelial Cell Monolayers. In the course of studies on virus maturation in MDCK cells, we observed that monolayers infected with VSV contained large numbers of virus particles in the intracellular spaces beneath intact tight junctions. Since these junctions prevent the passage of large molecules, this observation suggested that VSV might be able to spread laterally and form plaques on MDCK monolayers in the presence of anti-VSV antibody, provided that VSV receptors are present on basolateral cell surfaces. In initial experiments designed to assess the effect of antibody on plaque formation, we observed that after 30 hr, VSV had produced large plaques on BHK21 monolayers lacking antiserum, but no plaques were detected on plates to which antiserum had been added (Fig. 1a). After two days VSV had produced numerous small plaques on MDCK monolayers in the presence or absence of antiserum (Fig. 1b). In the presence of antiserum, the VSV plaques were somewhat smaller in size, but the average plaque number was not reduced significantly in most experiments. In contrast to VSV, influenza virus, which does not bud beneath the tight junctions of MDCK cells, did not form any plaques in these cells in the presence of anti-influenza virus antiserum (Fig. 1c).

We also observed that VSV-infected MDCK monolayers overlaid with liquid maintenance medium lacking antibody formed plaque-like lesions during the first 24 hr post infection. Subsequently, secondary sites of infection were observed that spread rapidly, and the monolayer was destroyed. However, when anti-VSV antiserum was included in the liquid overlay, VSV produced plaques similar in number and size to those formed with agar in the overlay.

These results indicate that VSV receptors are present on the basolateral surfaces of MDCK cells, and that VSV infection can spread laterally through MDCK monolayers in the presence of neutralizing antibody. Influenza virus, and presumably other viruses which do not form beneath tight junctions, will not be protected from neutralizing antiserum, whereas VSV and presumably other viruses capable of maturation and adsorption at basolateral membranes are able to spread and form plaques in the presence of antibody. In other

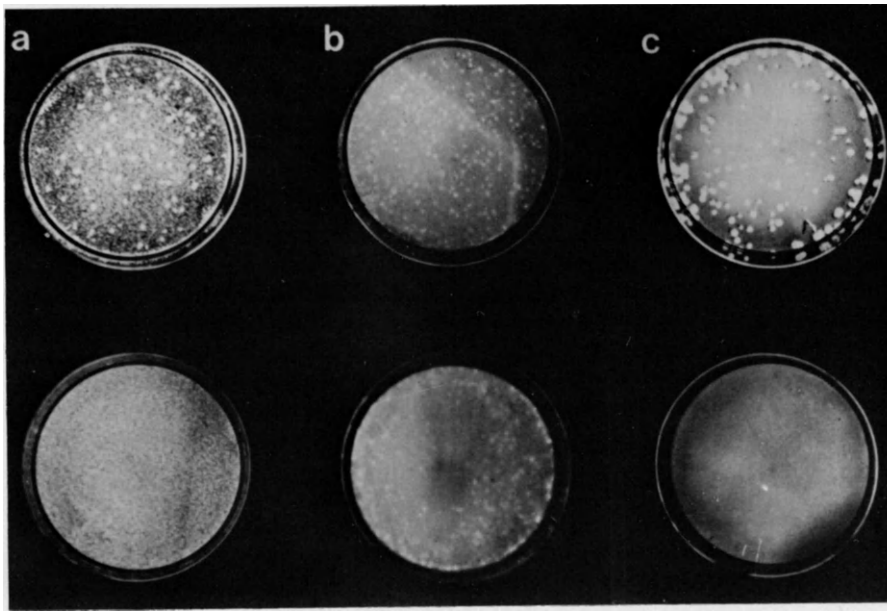

Fig. 1. Effect of antibody on plaque formation by VSV and influenza virus. Confluent monolayers were infected with VSV and incubated with agar overlays in the absence (upper row) or presence (lower row) of antiviral antiserum. In the case of influenza virus, 0.0005% trypsin was included in the overlay. Plates were fixed with 7% formaldehyde and stained with Giemsa stain at 2 days postinfection for BHK21 and 4 days postinfection for MDCK cells. (a) VSV plaques in BHK21 cells. (b) VSV plaques in MDCK cells (c) Influenza virus plaques in MDCK cells. From Roth and Compans (10).

cell lines where VSV matures at both apical and basolateral membranes, the presence of junctional complexes also permits the lateral spread of virus infection in the presence of heat inactivated antiserum, where inactivation of complement prevents possible immune cytolysis (results not shown). It will be of interest to determine whether restriction of viral glycoproteins to specific cell membrane domains might play a role in limiting the accessibility of viral antigens to the immune response during natural virus infections.

Phenotypic Mixing Between VSV and Influenza Virus in MDCK Cells. The assembly of myxo and paramyxovirus has also been reported to occur exclusively on apical membranes of MDBK bovine kidney cells, and that of VSV on basolateral membranes (6). These observations were difficult to reconcile with previous findings that mixed infection of MDBK cells with the paramyxovirus SV5 and VSV

yielded a large percentage of particles with VSV genomes, containing a mixture of envelope glycoproteins from each of the two virus types (13,14). Formation of such phenotypically mixed particles would require that glycoproteins of both viruses be present in the same membrane domains. We have observed strict polarity of influenza and VSV maturation sites in intact monolayers of MDCK cells, but in MDBK cells, VSV was sometimes observed budding at apical surfaces (10). Thus, we decided to determine if phenotypic mixing and pseudotype formation could occur between viruses which mature at distinct sites in MDCK cells. We have determined the time course in MDCK cells of formation of both phenotypically mixed VSV particles, bearing a mosaic of VSV and influenza virus glycoproteins, and of VSV(WSN) pseudotypes that are not neutralizable by VSV antibody. We have also examined the viral maturation sites in doubly infected MDCK cells in order to determine whether changes in glycoprotein transport or in virus maturation sites might occur.

Virus yields from mixed infections which had produced both wild type viruses were assayed for the presence of both phenotypically mixed VSV and VSV(WSN) pseudotypes. We have used the term "VSV(WSN) pseudotypes" to refer to VSV particles measured as VSV plaques resistant to pretreatment with amounts of anti-VSV serum shown to be sufficient for complete neutralization of control stocks (15,16). Presumably such plaques would be formed by virions containing a VSV core surrounded by an envelope bearing influenza virus glycoproteins and little or no VSV G protein. VSV particles containing some influenza virus glycoproteins, as determined by the total fraction of VSV plaques sensitive to antiserum specific for influenza virus, are termed "phenotypically mixed VSV". In 24 hr virus yields from doubly infected cells, percentages of pfu resistant to anti-VSV serum ranged from 0.5% to 3.4%, in experiments in which the interval between infections varied from 3 to 5 hr. In nine experiments where the interval between infections was 3 hr, the mean percentage of VSV(WSN) pseudotypes was 1.9%, and the mean percentage of phenotypically mixed VSV was 76.6% (Table 1). Premixing separately grown influenza virus and VSV did not protect VSV against neutralization by anti-VSV serum (data not shown).

The finding that phenotypic mixing occurs between VSV and influenza virus in MDCK cells indicates that the glycoproteins of both viruses are sometimes present in the same domain of the plasma membrane. This could occur by a change in glycoprotein transport in doubly infected cells, or by destruction of the junctional complexes separating the apical and basolateral membrane domains, followed by lateral diffusion in the plane of membrane and mixing of the glycoproteins. If directional transport were maintained, it seemed likely that the formation of phenotypically mixed VSV might not occur at early times during infection, although wild-type virus would

TABLE 1. PERCENTAGES OF PHENOTYPICALLY MIXED VIRUS AND VSV(WSN) PSEUDOTYPES IN VIRUS YIELDS[a]

Experiment	No Antiserum	+Anti-influenza Serum	Percent Phenotypically Mixed Virus	+Anti-VSV Serum	Percent Pseudotypes
1	3.0×10^7	8.5×10^6	71.6	3.5×10^5	0.4
2	1.5×10^7	5.1×10^6	65.3	1.9×10^5	1.3
3	3.4×10^7	6.5×10^6	81.0	4.9×10^5	1.4
4	2.6×10^7	5.9×10^6	77.7	4.8×10^5	1.8
5	1.8×10^7	2.7×10^6	85.2	2.0×10^5	1.1
6	2.6×10^7	8.1×10^6	69.1	7.0×10^5	2.8
7	2.7×10^7	5.7×10^6	79.9	8.0×10^5	3.0
8	3.7×10^7	8.5×10^6	77.3	6.2×10^5	1.7
9	2.8×10^7	5.7×10^6	80.0	6.7×10^5	2.4
Mean Values	2.7×10^7	6.3×10^6	77.6	5.0×10^5	1.6

(a) All samples are 24 hr yields from MDCK monolayers infected with 10 pfu/cell of influenza virus and superinfected with 10 pfu/cell VSV 3 hours later. Values are pfu/ml.

be produced. Therefore, we investigated the time course of phenotypic mixing and formation of pseudotypes in MDCK cells. In a one-step growth curve comparing total yields of VSV and phenotypically mixed VSV from doubly infected MDCK monolayers (Fig. 2A), it was observed that phenotypically mixed VSV does not appear as early as wild type virus, but rather is detected 1 or 2 hr later and in gradually increasing proportions. This result suggests that VSV and influenza viral glycoproteins are transiently segregated in the plasma membranes of doubly infected MDCK cells. In one-step growth curve comparing yields of VSV and VSV(WSN) pseudotypes, the production of pseudotypes was also observed to lag behind that of wild type virus (Fig. 2B). Both VSV and influenza virus were present in culture supernatants at 6 hr p.i. with VSV and VSV was produced in amounts which allow detection of 0.0001% pseudotypes; however, no pseudotypes were detected. In similar experiments, pseudotypes were not detected as cell associated virus at this time when doubly infected cells were scraped, frozen, and thawed. By 9 hr p.i. with VSV, over 10^4 pfu/ml of VSV(WSN) pseudotypes were detected. Although the exact time of appearance of pseudotypes was observed to vary, as did the onset of visible cytopathic effects in the monolayer and the final percentage of pseudotypes produced, in each of six independent experiments (Fig. 3A) the percentage of pseudotypes present in culture supernatants increased dramatically at later periods of infection. In fact, most VSV(WSN) pseudotypes were produced at very late times, after the cells had rounded. This delayed appearance of VSV(WSN) pseudotypes suggests that at early times p.i., VSV internal components are not able to form buds with influenza virus glycoproteins present at the apical surface. The period when VSV(WSN) pseudotypes become detectable corresponds to the first visible cytopathic effect in the monolayer, suggesting that opening of the junctional barriers between membrane domains followed by lateral diffusion of viral glycoproteins in the plane of the membrane may be prequisites for pseudotypes formation.

In order to investigate if the delay in pseudotype formation in MDCK cells is related to the phenomenon of polarity in virus maturation, we examined the time course of pseudotype formation in BHK21 cells. BHK21 fibroblasts do not form monolayers with cells connected by tight junctions, and polarity of virus maturation is not observed in these cells (Fig. 4). In contrast to double infections in MDCK cells, VSV(WSN) pseudotypes were formed in BHK21 cells as soon as wild type virus (not shown). Also, in BHK21 cells the proportion of pseudotypes present in culture supernatants did not increase at later times p.i. (Fig. 3B). These data suggest that in BHK21 cells the viral glycoproteins are never segregated in the plasma membrane, and that pseudotype formation can proceed with constant efficiency relative to the production of wild type VSV.

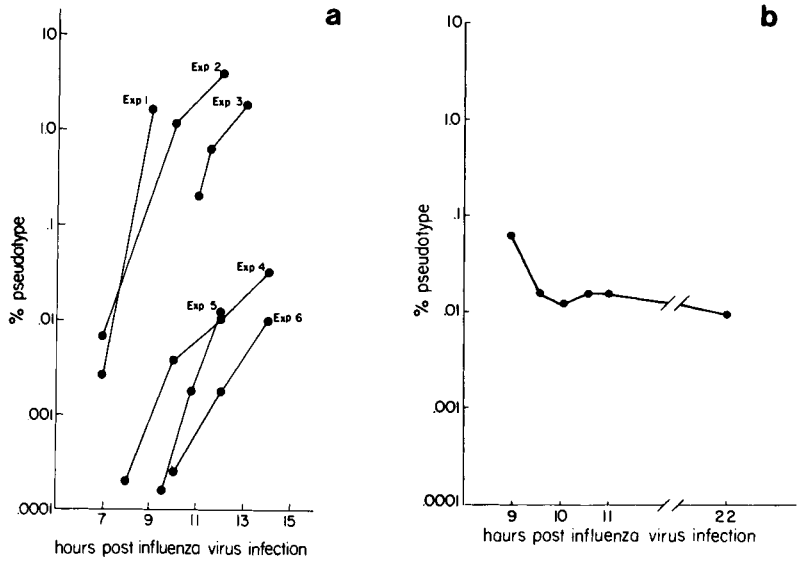

Fig. 2a. One step growth curve comparing total yields of VSV and phenotypically mixed VSV. MDCK monolayers were infected with 6 pfu/cell of influenza virus. After 3-1/2 hr, the monolayers received an equal m.o.i. of VSV. After 30 min at 37°C, residual VSV inoculum was neutralized with anti-VSV serum. Data points represent means from a minimum of four separate experiments, and at least two plaque assays were performed for each sample. Bars represent 95% confidence levels. ●——● VSV; o- - -o VSV treated with a 1/10 dilution of anti-influenza virus serum.◇ ---◇ phenotypically mixed VSV(WSN). At 19½ hr p.i. with VSV, P 0.001 by a two sample T-test. From Roth and Compans (11).

Fig. 2b. One step VSV growth curves for MDCK cells superinfected with 10 pfu/cell of VSV at 3 hr after primary infection with 10 pfu/cell of influenza virus. Residual VSV inoculum was neutralized with anti-VSV serum ●——● Untreated VSV. o---o VSV(WSN) pseudotypes, detected as pfu resistant to treatment with a 1/100 dilution of anti-VSV serum. From Roth and Compans (11)

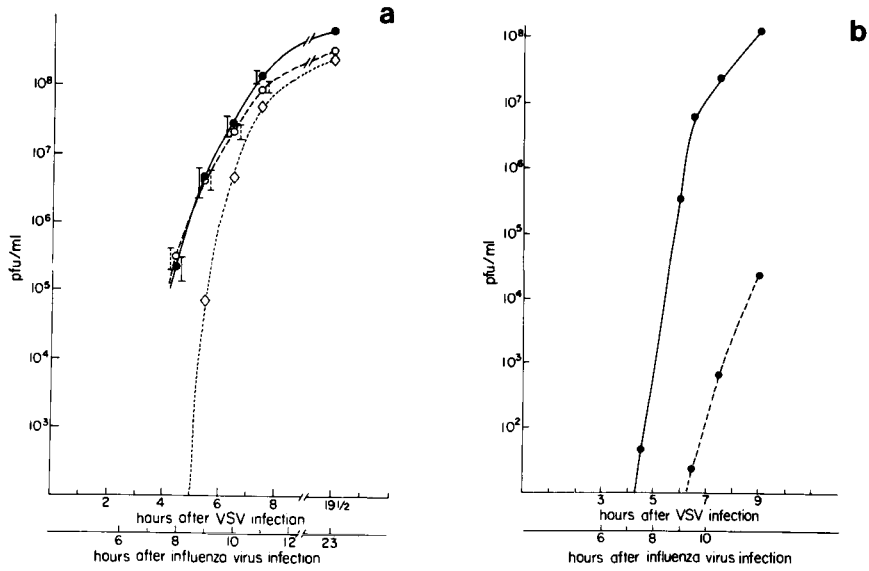

Fig. 3a. Progressive increase in percentages of VSV(WSN) pseudotypes after infection of MDCK cells. Infection with 10 pfu/cell of influenza virus was followed after 3 to 5 hr by superinfection with 10 pfu/cell of VSV. The results of six individual experiments are shown. From Roth and Compans (11).

Fig. 3b. Percentages of VSV(WSN) pseudotypes from BHK21 cells doubly infected as described in (a), plotted as a function of time after infection. Data represent the averages from 3 experiments. From Roth and Compans (11).

When thin sections of doubly infected MDCK cells were examined at early times p.i., polarity of maturation sites for both influenza virus and VSV appeared to be maintained (Fig. 5a). We did not usually detect altered maturation sites for either virus until 12 hr p.i. with influenza virus, a period later than we were able to detect pseudotypes by the more sensitive plaque assays. In cells where we did observe altered maturation sites (Fig. 5b), we also observed frequent disruption of junctional elements. These observations indicate that polarity of maturation continues during early periods in doubly infected cells, and is gradually lost after tight junctions have opened and the cells begin to round.

When doubly infected MDCK cells were treated with 3 mM EGTA from 5 to 6 hr p.i. with influenza virus, strict polarity of

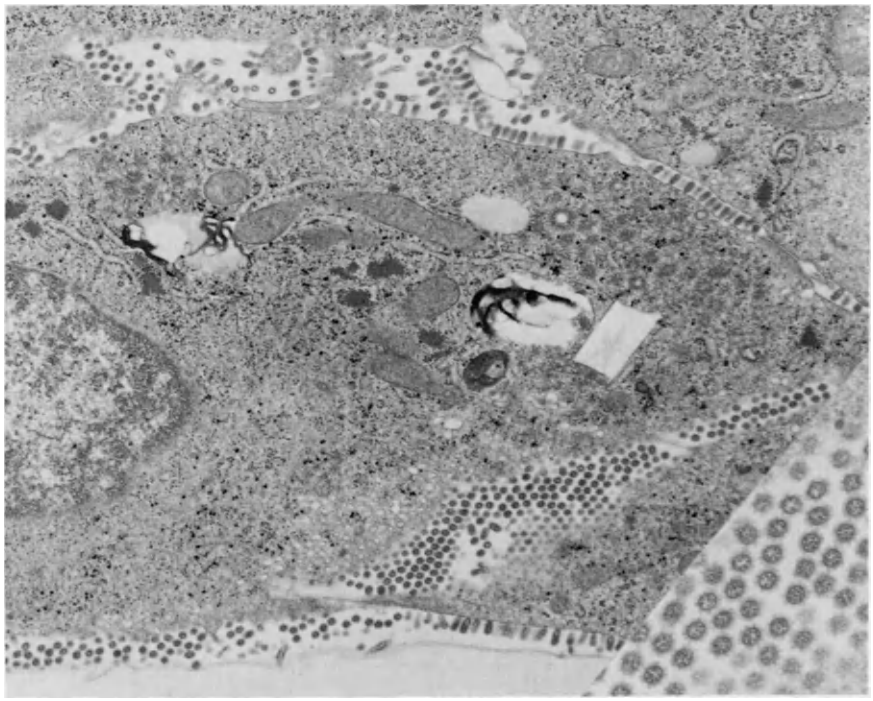

Fig. 4. Electron micrograph of BHK21 cells 13 hr post infection with A/WSN influenza virus. Virus formation is occurring all around the cell surface. The insert shows particles cut in cross section. Magnification: X 17,500; insert X 52,500.

maturation was not observed although the majority of each virus type remained associated with the normal membrane domain. In other experiments, cells were plated at low density shortly after superinfection with VSV. Most cells did not form tight junctions with neighboring cells although a majority were able to resume a normal flattened morphology several hours after plating. These cells were fixed for electron microscopy at 10 hr p.i. with influenza virus, and examination of thin sections revealed a complete loss of polarity of maturation (Fig. 5c). Therefore, treatments which increase cell rounding facilitate the loss of polarity of virus maturation.

Effects of Monensin on VSV and Influenza Virus Maturation in MDCK Cells. Ionophores that have an affinity for monovalent cations have been found to block the secretion of proteins and glycoproteins in a variety of eukaryotic cells (17-20). The inhibition of secretion by monensin, a linear polyether with a high affinity for Na^+ ions (21), is accompanied by a pronounced morphological change in the Golgi apparatus, with accumulation of dilated cytoplasmic vacuoles (18,20).

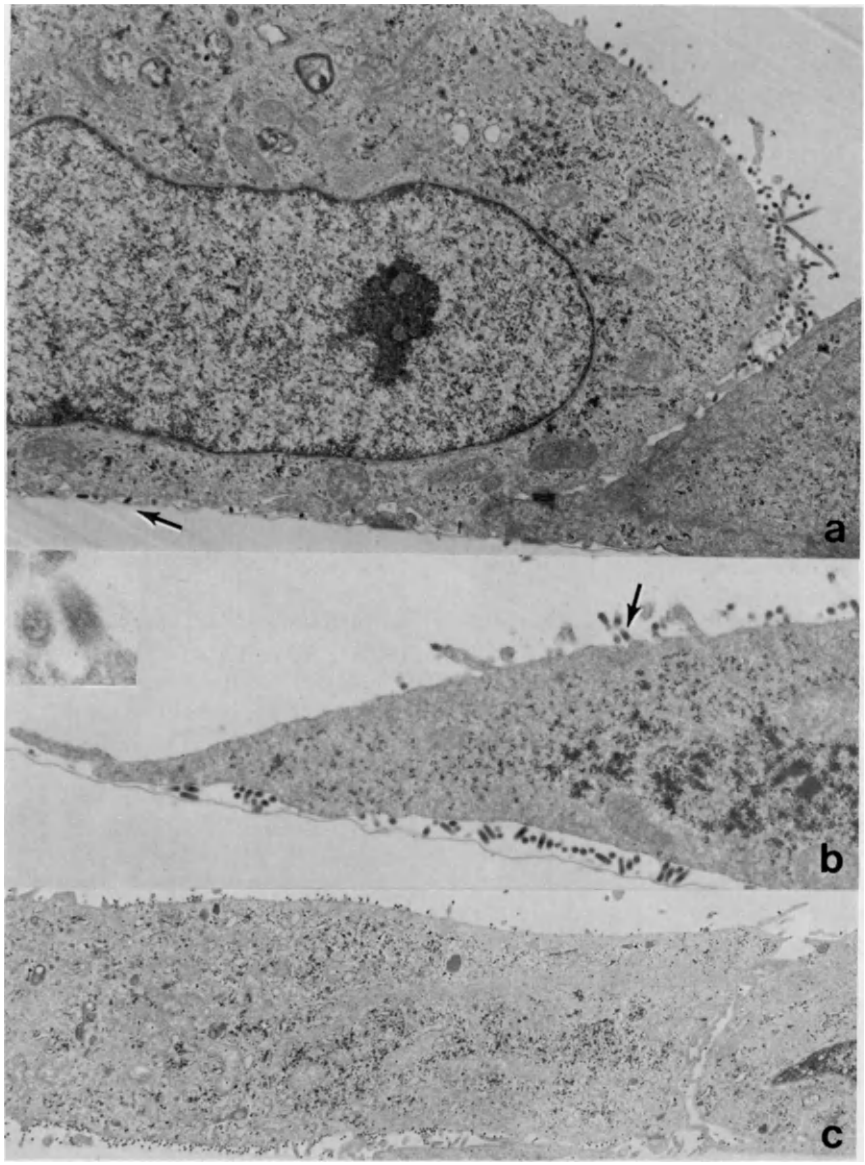

Fig. 5. Polarity of maturation in doubly infected MDCK cells: (a) 12 hr p.i. influenza virus is at apical, VSV (arrow) only at basolateral surfaces. X 8,000. (b) Altered maturation of VSV is seen in a doubly infected cell with a free lateral surface 12 hr p.i. X 13,000. Insert shows a VSV particle (arrow) budding apically. X 82, 500. (c) Cell replated after infection shows influenza virus at all surfaces 10 hr p.i. X 4,600.

It has been proposed that monensin affects intracellular transport at the level of the Golgi complex by blocking the release of secretory vesicles from Golgi membranes that become dilated as an effect of the drug (19). Because of the previous results indicating that different pathways are involved in transport of VSV and influenza virus glycoproteins to cell surfaces, we sought to determine whether the replication of influenza virus and VSV can be differentially affected by monensin.

A striking difference was found between the effect of monensin on the yields of infectious influenza viruses and VSV from infected MDCK cells. As shown in Fig. 6, the infectivity titers of VSV were markedly decreased with increasing concentrations of monensin. At a 10^{-6} M monensin concentration, the yield of VSV plaque-forming units was reduced by over 90%, and a reduction of over 4 logs in infectious virus yield was obtained with the highest concentration of monensin tested (10^{-4} M). In contrast, monensin had little or no effect on influenza virus yields in parallel experiments. At a 10^{-6} M concentration of monensin, the virus titer obtained was similar to that from the control cells. Even at a 10^{-4} M monensin concentration, less than a 3-fold decrease in yield of infectious influenza virus was obtained.

These results, together with previous observations (19-21) suggest that monensin blocks the transport of VSV glycoproteins to plasma membranes. When monensin-treated cells were examined by immunofluorescence with rhodamine-conjugated anti-VSV G protein IgG, a brilliant fluorescence was observed along the plasma membrane of cells not treated with monensin, while monensin-treated cells showed no fluorescence above background levels (12). In both instances, however, intracellular antigens were present. These observations indicate that VSV G protein is synthesized but is not transported to the cell surface in the presence of monensin. Influenza viral antigens were readily observed, however, on surfaces of monensin-treated cells.

The difference in the effect of monensin on replication of VSV and influenza virus was also clearly evident when thin sections of infected cells were examined by electron microscopy. VSV-infected MDCK cells treated with 10^{-5} M monensin contained dilated cytoplasmic vesicles, similar to the structures previously described in other monensin-treated cells (19,22). In most cells, these vesicles were localized near the basolateral surfaces. In some instances, these cytoplasmic vesicles contained VSV particles and other electron-dense material (Fig. 7A). Virions were rarely seen forming at the basolateral membrane. Numerous dilated cytoplasmic vesicles were also observed in influenza virus-infected MDCK cells treated with 10^{-5}M monensin (Fig. 7B). However, no influenza virions were observed within these structures. Numerous influenza virions were found budding at the free apical surfaces, as seen in infected MDCK cells not treated with monensin. In some instances, some virus

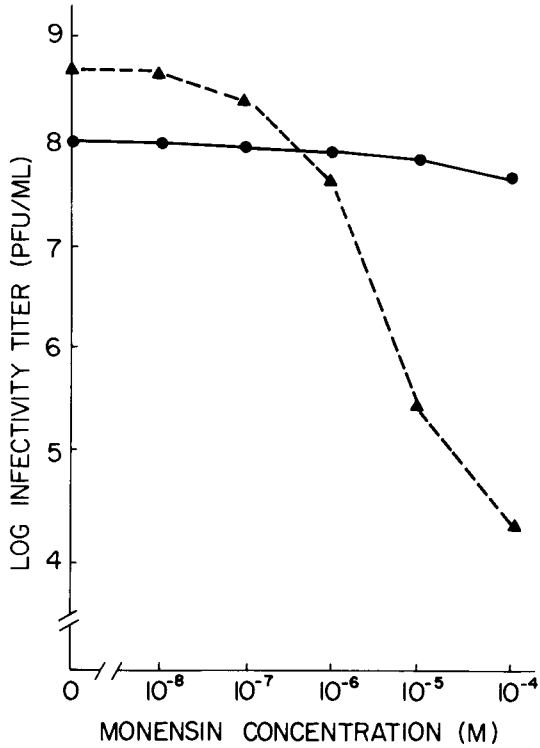

Fig. 6. Effect of monensin on the yields of infectious influenza virus and VSV in MDCK cells. Monensin was added post-adsorption to confluent MDCK monolayers infected with 10 plaque forming units per cell of influenza virus or VSV. Culture fluids were harvested 24 hr postinfection. Infectivity titers were determined by plaque assays on MDCK cells for influenza virus and on BHK21 cells for VSV. Influenza virus titers (o—o), VSV infectivity titers (▲---▲). From Alonso and Compans (12).

particles were observed at the basolateral membrane as well; however, this was also observed when controls not treated with monensin were examined at similar time points, indicating that strict polarity of maturation was lost at later times post-infection.

DISCUSSION

Our results demonstrate that phenotypic mixing and pseudotype formation occur in MDCK cells doubly infected with influenza virus, which buds from apical surfaces, and VSV, which matures basolaterally. Under one-step growth conditions, these phenomena occur principally at late times, following the appearance of cytopathic

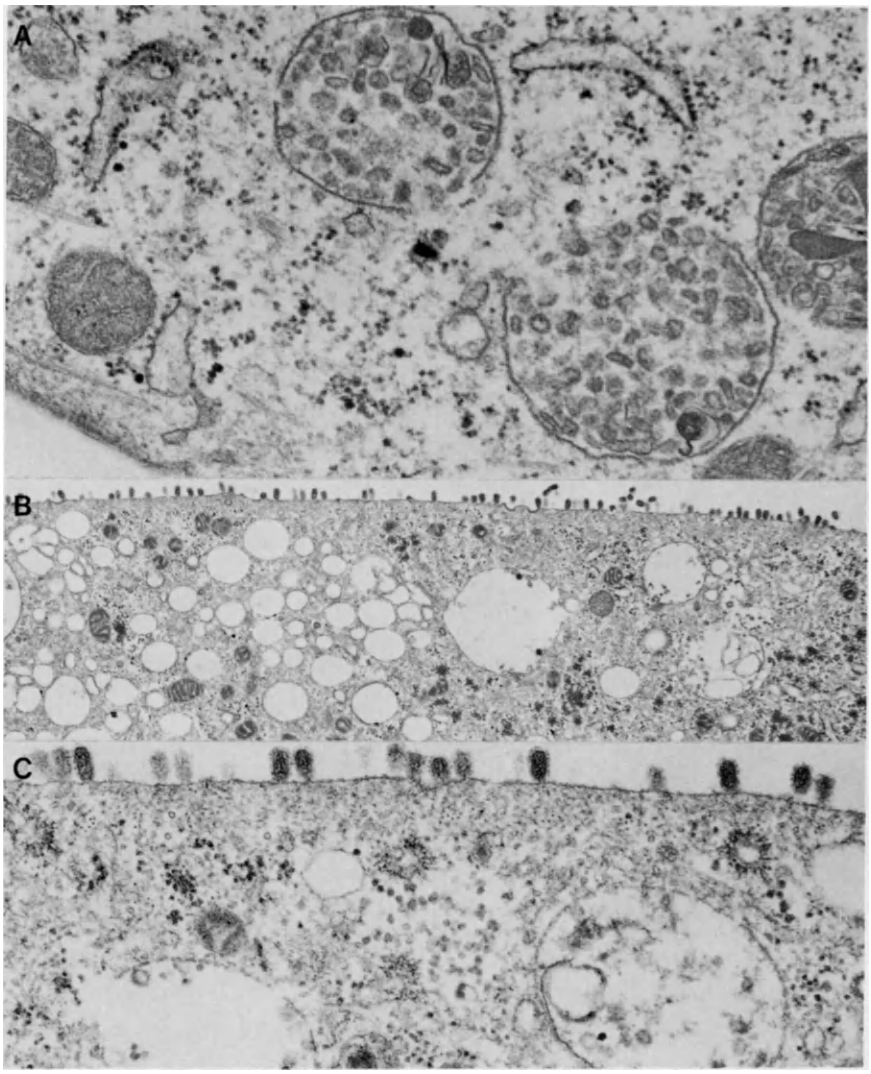

FIG. 7. A. VSV-infected MDCK cell treated with 10^{-5} M monensin (MOI=50). A cell at 9 hr postinfection shows cytoplasmic vesicles containing VSV particles and some electron-dense material. (X50,000). B. Electron micrograph of an MDCK cell infected with 10 PFU/ml of influenza virus and treated with 10^{-5} M monensin. This cell, harvested 12 hr postinfection, shows numerous dilated cytoplasmic vesicles which appear empty. Many budding influenza virions are observed at the apical surface (X13,200). C. A higher magnification view of influenza virus particles budding from the apical surface of a monensin-treated cell (X45,000).

effects in the monolayer, and in every case there is a significant lag behind the appearance of wild type viruses. The data obtained are consistent with a model for VSV morphogenesis in which the site of insertion of the viral glycoprotein determines the maturation site (7) and the glycoprotein is required at least transiently for budding (23, 24). It is likely that the factors limiting VSV(WSN) pseudotype formation in MDCK cells at early times p. i. are the directional transport of viral glycoproteins which segregates the two types of virus glycoproteins at the plasma membrane, and the requirement for at least small amounts of G protein for a nucleation event in assembly with VSV cores. We found that up to 77% of the VSV particles produced during double infection with influenza virus carried enough influenza glycoproteins to be neutralized by anti-influenza serum, indicating that in MDCK cells, VSV readily incorporates influenza virus glycoproteins into its envelope. This finding is in agreement with reports that VSV will incorporate glycoproteins of a wide variety of virus types into its envelope (16,25,26). MDCK cells exhibit hemadsorption and produce both influenza virus and wild type VSV before phenotypically mixed VSV or VSV(WSN) pseudotypes can be detected as either cell associated or released virus. Thus, influenza virus glycoproteins are present in the apical membrane of MDCK cells and VSV ribonucleoprotein cores and matrix protein are present intracellularly 8 hr p.i. with influenza virus in a typical experiment. If there were no requirement for the presence of G protein for formation of VSV in MDCK cells, then VSV(WSN) pseudotypes might be expected to form at the apical membrane during this period and VSV(WSN) pseudotypes might be present in significant quantities relative to VSV particles with mosaic envelopes. The fact that we did not detect VSV(WSN) pseudotypes at this time, and that VSV(WSN) pseudotypes were found to be 70 to 700 fold less common than phenotypically mixed VSV in the total virus yield, indicates that some VSV G protein is probably required for virus assembly.

At later times during single infections of MDCK cells with VSV or influenza virus, absolute polarity of virus maturation sites and directional transport of glycoproteins have been observed to disappear (9; our unpublished results) although a clear preference of each virus for its normal membrane domain is seen until late in infection when distinctions between those domains become obscure as the cell rounds up and dies. The results reported here indicate that directional transport of viral glycoproteins is a stable characteristic of singly and doubly infected cells, and probably continues after the destruction of functional elements responsible for maintaining distinct membrane domains. Thus the virus infected MDCK cells should prove to be very useful in studying the mechanisms by which glycoproteins are directionally transported in epithelial cells.

The present results clearly demonstrate that the pathways of transport and insertion of influenza virus and VSV glycoproteins into the plasma membrane of MDCK cells differ with respect to monensin

sensitivity. Infectivity titers of VSV are markedly reduced upon treatment of VSV-infected cells with monensin, although all viral proteins are synthesized. The G protein, however, does not appear to reach the cell surface, as was also observed by Johnson and Schlesinger (21) in two other cell types. Surprisingly, influenza virus yields in MDCK cells were unaffected or only slightly affected at the same concentrations of monensin. The schematic diagram in Fig. 8 depicts our interpretation of the effects of monensin on transport of viral glycoproteins. In VSV-infected MDCK cells treated with monensin, dilated vacuoles similar to those found in other monensin-treated cells were observed, which are thought to result from dilation of the Golgi complex (19,21). The observed accumulation of VSV particles in vacuoles may be a result of budding of virus into cytoplasmic vesicles, as has been observed with Sindbis virus in the presence of the ionophore (21). In contrast, influenza virus-infected cells treated with the same concentrations of monensin showed numerous virions budding at the free apical surfaces, as in untreated cells. Dilated cytoplasmic membranes were also observed in these cells but no influenza virus particles were found inside these structures. Since the production of infectious influenza virus requires the presence of two functional glycoproteins, hemagglutinin and neuraminidase, at the plasma membrane, it is clear that the transport of influenza virus glycoproteins to the cell surface continues to occur. A recent report indicates that newly-synthesized VSV glycoproteins are transported from the endoplasmic reticulum to the plasma membrane via clathrin-coated vesicles (27). It is possible that monensin blocks exit of these coated vesicles from the Golgi complex. There is no information as yet on the composition of structures involved in mediating the transit of influenza virus glycoproteins to the cell surface, but the present results suggest that they are transported by vesicles whose formation is insensitive to monensin.

Other viral and cellular membrane proteins also exhibit polarized distributions in epithelial cells (9,28). It will be of interest to determine whether the differences in response to monensin observed with VSV and influenza viral glycoproteins reflect general properties of proteins localized in apical vs. basolateral membrane domains. The use of ionophores that selectively block transport of one class of membrane proteins should also aid in elucidating the mechanisms by which membrane glycoproteins are transported from the Golgi complex to specific cell surface domains.

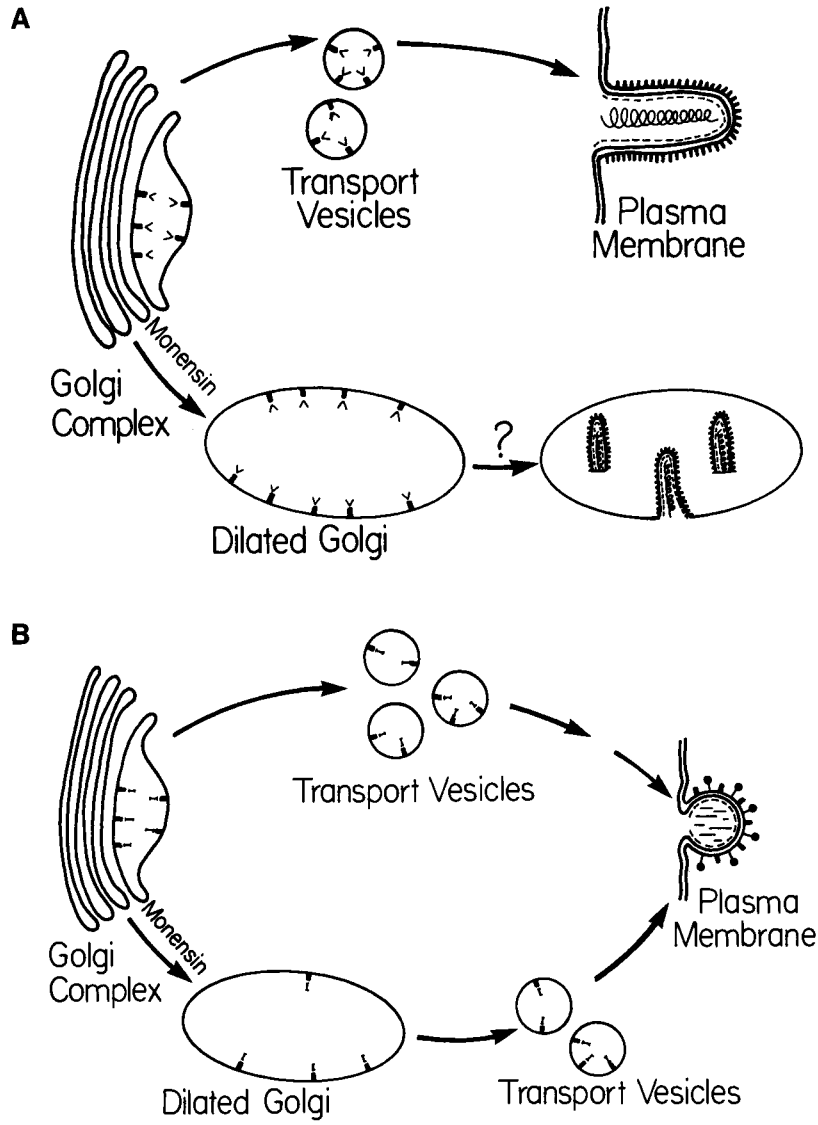

Fig. 8. Schematic diagram of the possible steps in intracellular transport of viral glycoproteins in MDCK cells and the effects of monensin on these processes.
(A) Normally, VSV G protein is transported from the Golgi complex via clathrin-coated transport vesicles to the cell surface where the virus buds. Treatment with monensin leads to dilation of Golgi complex membranes and a block in the intracellular transport of VSV

Fig. 8, ctd. glycoproteins. Possibly, since the G protein is restricted to the membrane of the dilated Golgi, budding of VSV virions may occur at this site.
(B) Influenza virus glycoproteins are also thought to be transported from the Golgi apparatus to the plasma membrane by transport vesicles. Monensin treatment results in the dilation of the Golgi elements. However, transport of influenza virus glycoproteins continues to occur, probably in transport vesicles whose formation is resistant to monensin.

REFERENCES

1. Cereijido, M., Robbins, E. S., Dolan, W. J., Rotunno, C. A. and Sabatini, D. D. 1978. J. Cell. Biol. 77, 853-880.
2. Leighton, J., Estes, L. W., Mansukhan, S. and Brada, Z. 1970. Cancer 26, 1022-1028.
3. Lever, J. E. 1979. Proc. Natl. Acad. Sci. USA. 76, 1323-1327.
4. Misfeld, D. S., Hamamoto, S. T. and Pitelka, D. R. 1976. Proc. Nat. Acad. Sci. USA 73, 1212-1216.
5. Rindler, M. J., Chuman, L. M., Shaffer, L. and Saier, M. H. 1979. J. Cell Biol. 81, 635-648.
6. Rodriguez Boulan, E. and Sabatini, D. D. 1978. Proc. Natl. Acad. Sci. USA 75, 5071-5075.
7. Roth, M. G., Fitzpatrick, J. P. and Compans, R. W. 1979. Proc. Natl. Acad. Sci. U.S.A. 76, 6430-6434.
8. Tkacz, J. S. and Lampen, J. O. 1975. Biochem. Biophys. Res. Commun. 65, 248-257.
9. Rodriguez Boulan, E., and M. Pendergast. 1980. Cell 20: 45-54.
10. Roth, M. G., and R. W. Compans. 1981. J. Virol. 35: 547-550.
11. Roth, M. G. and R. W. Compans. 1980. Submitted for publication.
12. Alonso, F. V. and R. W. Compans. 1981. J. Cell Biol., in press.
13. Choppin, P. W., and R. W. Compans. 1970. J. Virol. 5: 609-616.
14. McSharry, J. J., R. W. Compans, and P. W. Choppin. 1971. J. Virol. 8: 722-729.
15. Rubin, H. 1975. Virology 26: 270-276.
16. Zavada, J. 1976. Arch. Virol. 50: 1-15.
17. Ledger, P. W., N. Uchida, and M. L. Tanzer. 1980. J. Cell Biol. 87: 663-671.
18. Tajiri, K., N. Uchida, and M. L. Tanzer. 1980. J. Biol. Chem. 365: 6036-6039.
19. Tartakoff, A. M., and P. Vassalli. 1977. J. Exp. Med. 146: 1332-1345.
20. Uchida, N., H. Smilowitz, and M. L. Tanzer. 1979. Proc. Natl. Acad. Sci. U.S.A. 76: 1868-1812.
21. Johnson, D. C., and M. J. Schlesinger. 1980. Virology 103: 407-424.

22. Kaariainen, L., K. Hashimoto, J. Saraste, I. Virtanen, and K. Penttinen. 1980. J. Cell Biol. 87: 783-791.
23. Knipe, D. M., D. Baltimore, and H. F. Lodish. 1977. J. Virol. 21: 1128-1139.
24. Witte, O. N., and D. Baltimore. 1977. Cell 11: 505-511.
25. Weiss, R. A. 1980. In. Rhabdoviruses. D. H. L. Bishop (ed.), CRC Press, West Palm Beach, FL Vol. 3: pp. 51-65.
26. Weiss, R. A., and P. L. P. Bennett. 1980. Virology 100: 252-274.
27. Rothman, J. E., and R. E. Fine. 1980. Proc. Natl. Acad. Sci. U.S.A. 77: 780-784.
28. Louvard, D. 1980. Proc. Natl. Acad. Sci. U.S.A. 77: 4132-4136.

ANTIGENIC CARBOHYDRATE DETERMINANTS ON INFLUENZA HEMAGGLUTININ

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I. ABSTRACT

The specificity of antibodies elicited by the carbohydrate on chicken egg grown influenza virus and by chorioallantoic membranes from uninfected eggs has been examined. Antigenic carbohydrate determinants have been located on the oligosaccharide side-chains attached at residues 8 and 22 of the HA_1 polypeptide from two Hong Kong viruses and at residue 11 of an Asian HA_1. The oligosaccharide unit at position 154 in the HA_2 of both subtypes is also antigenic. The composition and attachment sites of these side-chains are compared.

II. INTRODUCTION

The hemagglutinin (HA) of influenza virus consists of approximately 19% (by weight) carbohydrate (1,2). From the

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recent x-ray crystallographic data of Wilson et al (3) the majority of this component is found surrounding the lower half of the timer nearest nearest the membrane. The function of the carbohydrate is not clear although it is thought to be necessary in protection of the newly synthesized HA against degradation by proteolytic enzymes (4). This is consistent with the observation that 10 potential tryptic and chymotryptic cleavage sites on each monomer are covered by carbohydrate (3). Another important role may be in stabilizing the structure of the molecule since carbohydrate has been found at the interface between HA subunits and at intra- and inter- chain contact regions (3).

Unlike in other viral surface glycoproteins, such as those from Semliki Forest virus (5), preliminary findings have indicated that the carbohydrate on influenza HA does not play a direct role in inducing the conformation required for antigenicity of the polypeptide since non-glycosylated HA can react equally well with anti-viral antibodies as does fully glycosylated HA (6). On the other hand, as pointed out by Wiley et al (7), the immunogenic potential of polypeptide determinants may be influenced by carbohydrate as it can effect the accessibility of various amino acid residues.

Under certain circumstances the carbohydrate itself can be immunogenic and antigenic, a fact that has been recognized for many years (8,9,10). Antibodies raised in rabbits against chicken egg-grown virus show broad cross reactivity with different strains grown in the same host by virtue of common carbohydrate determinants. Furthermore, antibodies elicited in rabbits against chorioallantoic membranes or allantoic fluid from uninfected eggs (host antigen) can bind to chicken egg-grown influenza virus, an interaction also mediated through carbohydrate.

The carbohydrate on HA is in the form of branched oligosaccharide side-chains that are attached to the polypeptide backbone through N-glycosidic linkage to asparagine residues (11,12). Potential sites for glycosylation occur only when the asparagine residue is in the tripeptide sequence Asn-X-Ser/Thr (13). The process is mediated by host cell enzymes, commencing with the transfer of N-acetylglucosamine and mannose 'cores' from lipid precursors to newly synthesized HA chains (14). Subsequent modification of the cores results, in some instances, in the addition of galactose and fucose residues.

In recent years many studies have been carried out in which small peptides, containing single oligosaccharide side chains, have been isolated following proteolytic digestion of the HA (15-19). The number and composition of side-chains from different virus strains grown in different hosts have been determined and their degree of sulphation, enzyme sensitivities and lectin binding properties examined. In this study we determine the relationship of the immunogenic determinants, common to different virus strains grown in the same host, to those determinants capable of interaction with antibodies raised against host antigen. The technique of fragmentation of the HA by proteolytic cleavage is then used to locate the oligosaccharide side-chains that possess these determinants.

III. METHODS

The influenza virus 'recombinants' Jap_H-Bel_N, Mem_H-Bel_N and $shearwater_H$-Bel_N bearing the HAs of A/Japan/305/57 (H2), A/Memphis/102/72 (H3) and A/shearwater/E. Aust/72 (Hav6) respectively and the NA of A/Bellamy/42 (N1) together with Bel (A/Bellamy/42 (HON1) virus) and X-31 (a 'recombinant' of A/Aichi/68 (H3N2) and A/PR/8/34 (HON1) bearing the H3 hemagglutinin) were used in this study.

Antiserum, IgG and host antigen production have been described in full elsewhere (20,21) as have the methods for HA and HA_1 preparation, radiolabelling, amino acid sequencing, peptide fragmentation and high voltage paper electrophoresis (22-24). Cyanogen bromide digests of ^{14}C-S-carboxymethylated HA_1 were separated on a Sephadex G-100 column in 50% formic acid and tryptic digests on two Sephadex G-50 fine columns connected in series (23). Column fractions were monitored by absorbance at 280 or 230 nm and ^{14}C-radioactivity. Aliquots were also examined for carbohydrate antigenic activity by their ability to inhibit the binding of anti-(host antigen) IgG to radioiodinated homologous HA_1 (25). The percentage inhibition obtained was converted to arbitrarily defined inhibitory units by reference to a standard curve in which unlabeled homologous HA_1 was the competitive inhibitor (25). Radioimmunoassays in which unlabeled antigen was used to coat the wells of a polyvinyl microtiter plate were performed as previously described (26).

IV. RESULTS

A. Cross-Reactivity of HA From Influenza Viruses of Different Subtypes

Immunoglobulin G from antisera prepared in rabbits against the 'recombinant' viruses Mem_H-Bel_N (H3N1) and Jap_H-Bel_N (H2N1) were titrated in wells of a polyvinyl plate coated with the isolated HA_1 from these strains and also from Bel (HON1) and $shearwater_H$-Bel_N (Hav6N1) viruses. The results (Figure 1) indicate that both viruses are capable of eliciting antibodies that can bind to the heterologous subtypes.

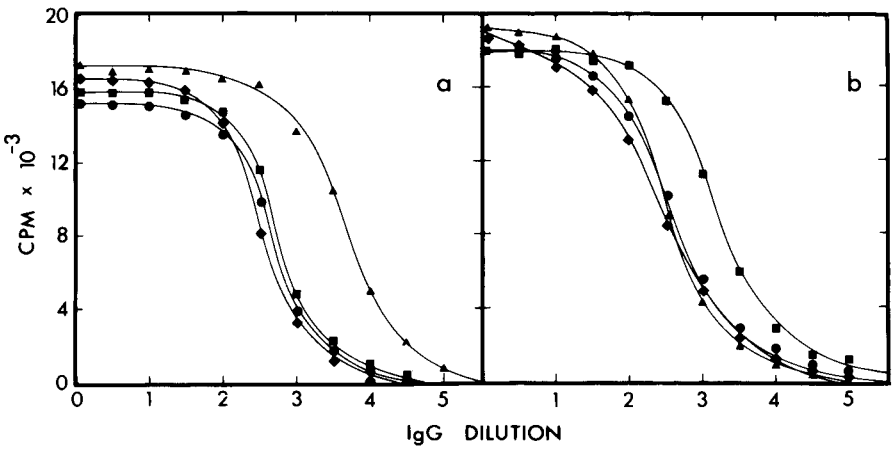

FIGURE 1. Binding of anti-(Mem_H-Bel_N) IgG (a) and anti-(Jap_H-Bel_N) IgG (b) to radioiodinated Mem (▲), Jap (■), Bel (●) and Shearwater (◆) HA1s.

In this assay system the lateral displacement between parallel curves provides a measure of the difference in titre that the antibody exhibits for different antigens whereas differences in slope reflect different affinities of interaction. It can be seen that antibodies directed against the cross-reactive carbohydrate comprise a substantial amount of the total reactivity against HA_1 and that the

amount of antibody capable of reacting with each heterologous strain is approximately the same. Furthermore, the similar slopes of the curves suggest that the cross-reactive antibodies have similar overall affinities for the determinant(s) that they are recognizing on each of the subtypes.

In order to determine whether the same population of antibodies reacts with each of the heterologous subtypes or whether different subpopulations are involved, those antibodies cross-reacting with one of the strains were removed and the remaining IgG tested for residual activity against the other strains. Passage of each anti-viral IgG through a column of shearwater HA, covalently attached to Sepharose 4B, will remove all antibodies against antigenic carbohydrate common to both the immunizing virus and shearwater HA. The effluent anti-(Mem_H-Bel_N) and anti-(Jap_H-Bel_N) IgG preparations (Figures 2a & b respectively) after such treatment, were tested for their ability to react with the four HA_1 subtypes.

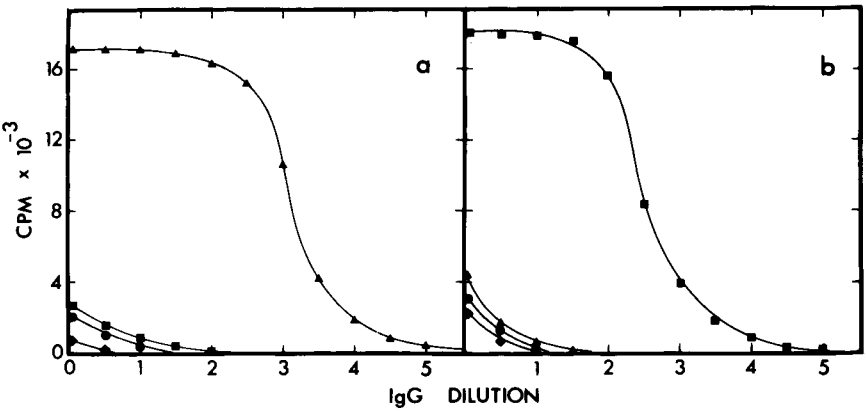

FIGURE 2. Binding of shearwater HA-adsorbed anti-(Mem_H-Bel_N) IgG (a) and anti-(Jap_H-Bel_N) IgG (b) to radioiodinated Mem (▲), Jap (■), Bel (●) and shearwater (◆) HA_1s.

All significant reactivity against shearwater HA was removed after adsorption and virtually no additional activity against the other heterologous viruses remained. Moreover, when the antibody that bound to shearwater HA was eluted and titrated against each HA_1 (Figure 3) cross-reaction was

again observed. The apparent subtle differences in affinity and titre under these conditions may reflect small differences in the number and/or structure of the determinants.

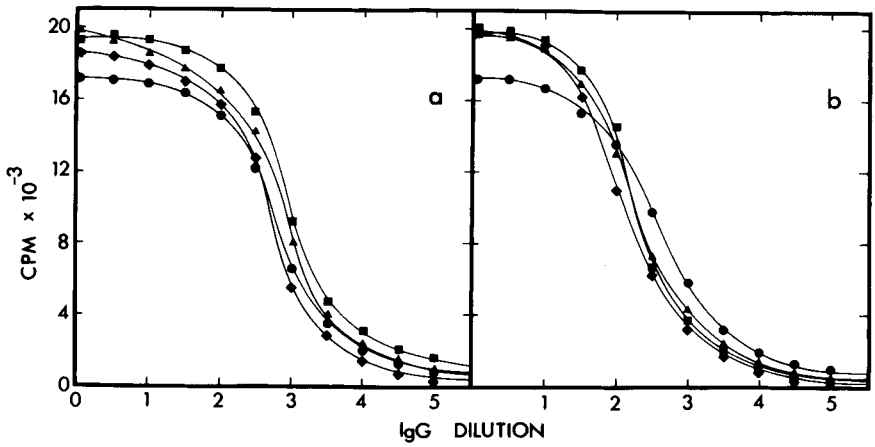

FIGURE 3. Titration of the cross-reactive antibodies from anti-(Mem_H-Bel_N) IgG (a) and anti-(Jap_H-Bel_N) IgG (b) against Mem (▲), Jap (■), Bel (●) and shearwater (◆) HA_1s.

B. Relationship of Cross-Reactive Carbohydrate Determinants to Host Antigen

Rabbit antibody to chick allantoic host antigen binds with a high and similar titer to each radiolabeled HA_1, indicating that one or more determinants are shared between the host antigen and HAs from each subtype grown in the same host. To establish whether these determinants are the same as those recognized when intact virus is used as the immunogen, the binding of anti-viral IgG to each HA_1 was also examined (i) following prior adsorption of the IgG with chorioallantoic membrane extract or lyophilized allantoic fluid from uninfected chicken eggs, covalently coupled to Sepharose 4B, and (ii) with a carbohydrate extract from chorioallantoic membranes present in the radioimmunoassay as a competitive inhibitor. Both of these treatments resulted in the abolition of anti-(Mem_H-Bel_N) and anti-(Jap_H-Bel_N)

IgG binding to heterologous HA_1s (results not shown). This indicates that all of the cross-reactivity can be attributed to carbohydrate and also that the antibodies in anti-viral IgG, responsible for the cross-reactivity between subtypes, do not recognize additional determinants to those also present on host antigen.

C. Localization of Antigenic Carbohydrate on Hong Kong HA_1

Sequential proteolytic cleavage was used to determine which of the oligosaccharide side-chains contain these cross-reactive determinants. Carbohydrate antigenicity was assayed by the ability of the glycopeptides to bind to anti-(host antigen) IgG. Reduced and alkylated HA_1, previously shown to have lost polypeptide antigenicity (27), was used as a convenient starting material for this investigation. Carboxymethylation with ^{14}C-iodoacetamide provided a radiolabel as an aid to monitoring peptides containing cysteine residues.

The HA_1 chain of both Mem and X-31 (A/Aichi/68) were examined. A comparison of these two Hong Kong strains was of interest because although the side-chains occur at the same positions on the polypeptide backbone in the two viruses, the compositions are not identical (17,18) as shown in Table 1. The position of the oligosaccharide side-chains on Mem and X-31 HA_1 and the location of fragments resulting from cyanogen bromide (CN), tryptic (T) and thermolytic (Th) cleavage of the molecule are shown in Figure 4.

The five fragments resulting from cyanogen bromide cleavage of reduced and alkylated Mem and X-31 HA_1, were readily separated by gel filtration (as shown for X-31 in figure 5) and the peaks identified by amino acid analysis (23). Aliquots from each of the fractions were examined for their ability to inhibit the binding of anti-(host antigen) IgG to radioiodinated HA_1. In both strains the inhibitory activity of the digest was exclusively located in the peak corresponding to CN1.

Pooled fractions containing CN1 were digested with trypsin and the resulting 10 peptides were separated by gel filtration (Figure 6). Examination of the fractions for inhibitory activity revealed that the contents of the first

TABLE 1. Carbohydrate Composition of the Oligosaccharide Side-Chains of MEM and X-31 HA

	Asparagine residue	Sugar composition (moles/mole peptide)				Carbohydrate type
		N-acetylglucosamine	Mannose	Galactose	Fucose	
Mem HA_1	8	4.1	4.1	4.8	1.8	N[a]
	22	3.7	2.1	2.2	1.3	N
	38	4.0	5.4	2.5	0.6	N
	81	2.9	2.5	2.1	0.2	N
	165	1.9	5.8			O
	285	2.0	5.1			O
Mem HA_2	154	4.0	2.6	2.2	1.1	N
X-31 HA_1	8	4.3	5.3	2.6	1.7	N
	22	4.3	3.3	2.3	1.9	N
	38	2.1	6.2	0.6	0.3	O
	81	3.8	3.4	3.1	0.9	N
	165	1.9	8.6			O
	285	1.9	4.4			O
X-31 HA_2	154a	3.9	3.9	2.3	1.5	N
	b	3.2	2.9	1.5	0.9	N

[a] N, N-acetyllactosamine type (containing all four sugar residues)
O, oligomannoside type (containing N-acetylglucosamine and mannose only)
Data from reference 17-18.

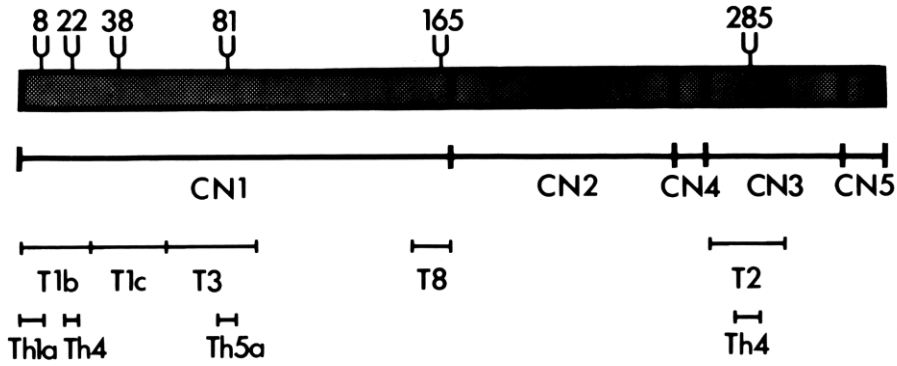

FIGURE 4. Oligosaccharide attachment sites and cyanogen bromide, tryptic and thermolytic peptides of H3 HA_1.

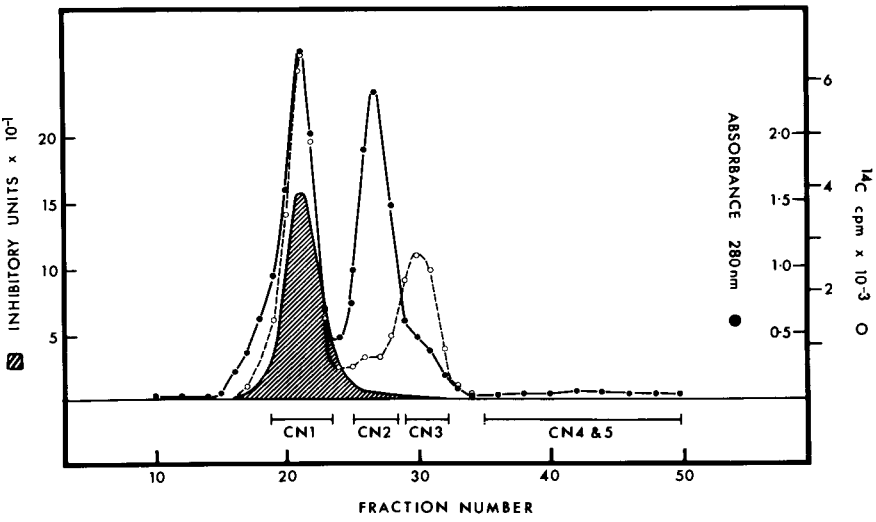

FIGURE 5. Separation of cyanogen bromide fragments of ^{14}C-S-carboxymethylated X-31 HA_1 by gel filtration.

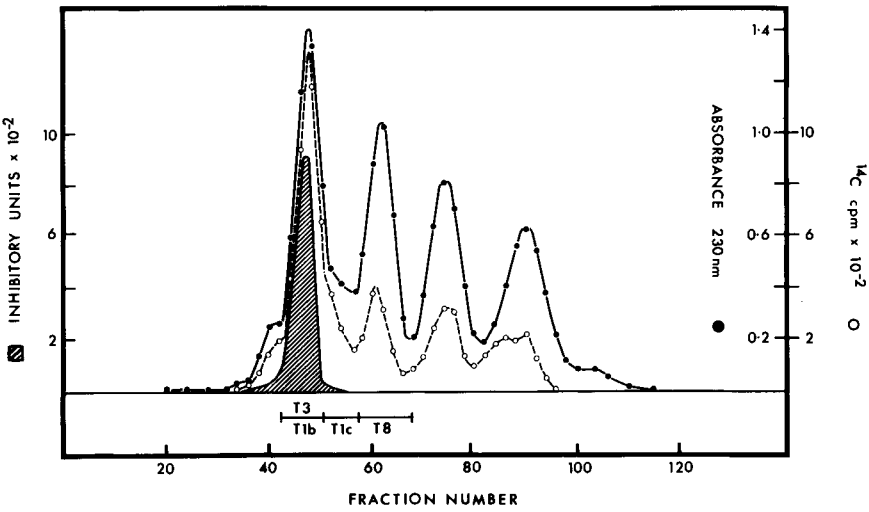

FIGURE 6. Separation of tryptic peptides of X-31 CN1.

major peak bound to anti-(host antigen) IgG. N-terminal and amino acid analysis indicated that this peak contained the peptides T1b (residues 1-27), which has carbohydrate at positions 8 and 22, and T3 (residues 58-90) which has carbohydrate at position 81. The other tryptic glycopeptides, T1c (residues 28-50, carbohydrate at Asn_{38}) and T8 (residues 157-168, carbohydrate at Asn_{165}) which eluted in fractions 51-57 and 58-67 respectively were unable to inhibit the binding of anti-(host antigen) IgG to HA_1.

Digestion of the material in fractions 43-50 with thermolysin yielded glycopeptides (shown in Figure 4) containing a single oligosaccharide side-chain: Th1a, residues 1-10, carbohydrate at Asn_8; Th4, residues 19-24, carbohydrate at Asn_{22}; and Th5a, residues 79-85 carbohydrate at Asn_{81}. These, together with peptides T1c (containing Asn_{38}), T8 (containing Asn_{165}) and the thermolytic peptide T2.Th4 (carbohydrate at Asn_{285}) were purified by high voltage paper electrophoresis and the full complement of isolated glycopeptides titrated in the inhibition assay (Figure 7).

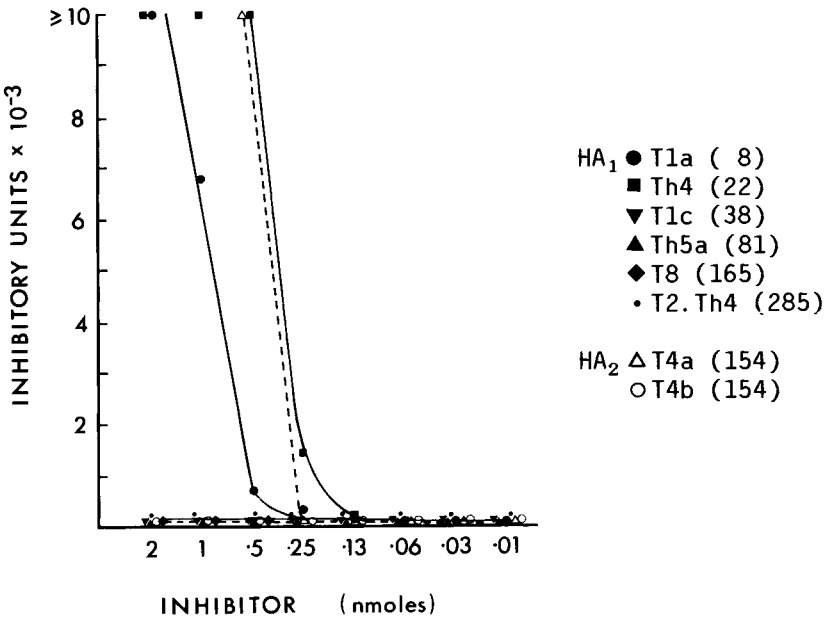

FIGURE 7. Inhibitory activity of glycopeptides derived from X-31 HA.

As shown in Figure 7 for X-31 HA, the major cross-reactive carbohydrate determinant was found to be the N-acetyllactosamine unit at Asn_{22} in glycopeptide Th4. Significant activity was also associated with the N-acetyl-lactosamine type carbohydrate unit at Asn_8 on peptide Thla. The thermolytic peptide Th5a failed to inhibit the binding of anti-(host antigen) IgG, and thus the N-acetyllactosamine unit at position 81 was not contributing to antigenic activity. Figure 7 also shows that, as predicted from testing fractions resulting from cyanogen bromide and tryptic cleavage, the oligosaccharide side-chains at positions 38, 165 and 285 do not react with anti-(host antigen) IgG. Glycopeptides isolated from Mem HA_1 gave the same pattern of reactivity. Therefore, in both Mem and X-31 HA_1 the antigenic carbohydrate side-chains are located at positions 8 and 22 in the amino acid sequence.

D. Localization of Antigenic Carbohydrate on Asian HA_1

The amino acid sequence deduced from the gene sequence of A/Japan/305/57 (H2) hemagglutinin showed that the HA_1 polypeptide possessed seven potential Asn-X-Ser/Thr glycosylation sites (28) at asparagine residues 10, 11, 23, 139, 164, 165 and 285. Peptide isolation and sequencing (28) have established that the asparagine residues at positions 10 and 139 do not carry carbohydrate but that residues 11, 23 and 285 do. Although it was known that additional carbohydrate was present at either 164, 165 or both, the exact residue of attachment was not known with certainty. From the homology between the sequence at residues 10-13 in Jap HA_1 and a similar sequence in β-haptoglobin it was suggested (29) that only the second asparagine residue (position 165) would carry the carbohydrate.

As the distribution of oligosaccharide side-chains on the HA_1 of H2N2 strains appears to be quite different to that of the H3N2 strains, it was of interest to determine whether the antigenic carbohydrate would be located at the N-terminal region of Jap HA_1 as was found in Mem and X-31 HA_1. Using similar cleavage and fractionation procedures to those used for Mem and X-31, glycopeptides were derived from the HA_1 of A/Japan/305/57 virus and tested for activity with anti-(host antigen) IgG.

Cyanogen bromide cleavage of Jap HA_1 yields eight fragments (30) designated CN1 to CN8 (see Figure 8) which

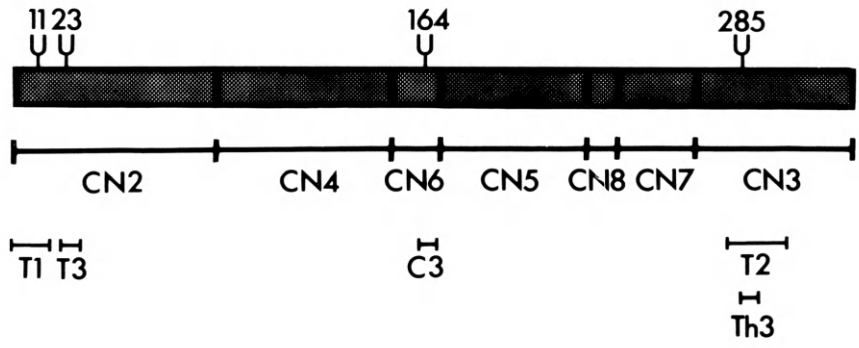

FIGURE 8. Oligosaccharide attachment sites and cyanogen bromide (CN), tryptic (T), chymotryptic (C) and thermolytic (Th) peptides of Jap HA_1.

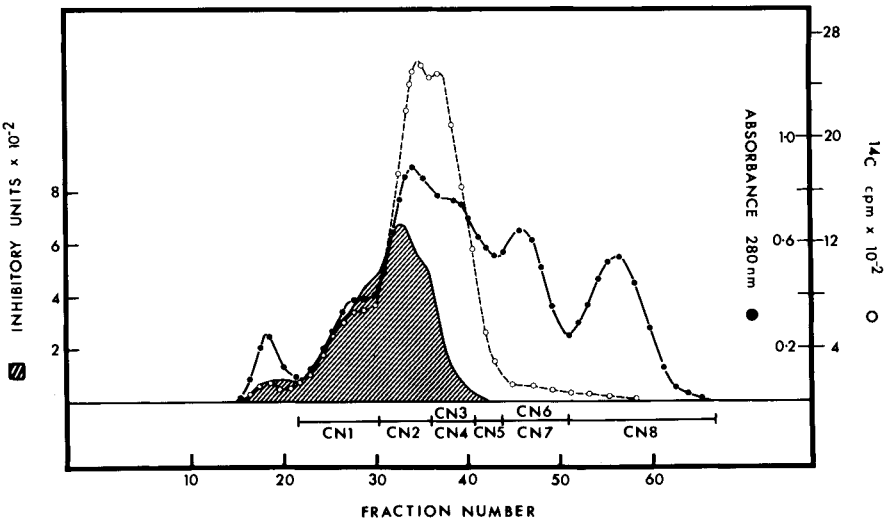

FIGURE 9. Separation of cyanogen bromide fragments of ^{14}C-S-carboxymethylated Jap HA_1.

may be partially separated by gel filtration (Figure 9). Screening the fractions for inhibitory activity revealed that antigenic carbohydrate was located in fractions 22-40. The shoulder occurring at fractions 22-29 contained the partial cleavage product CN1 (residues 1-146) while the major peak of inhibitory activity (fractions 30-36) was associated with fragment CN2 (residues 81-146). Both CN1 and CN2 contain oligosaccharide side-chains at Asn_{11} and Asn_{23} but some contribution to the antigenic activity by fragment CN3 (residues 265-324, carbohydrate at Asn_{285}) which lies in the neighboring fractions 36-42 cannot be ruled out. Fractions 45-52 which contain the glycosylated CN6 fragment (residues 147-171, carbohydrate at Asn_{164}) did not react with anti-(host antigen) IgG.

Tryptic cleavage of CN2 and separation on coupled Sephadex G-50 and G-25 columns gave the profile shown in Figure 10. The inhibitory activity was associated with the leading edge of the first peak which contained T1 (residues 1-15, carbohydrate at Asn_{11}). Glycopeptide T3 (residues 23-30) which has an oligosaccharide side-chain at Asn_{23}, was present in the shoulder at the trailing edge of the first peak (fractions 42-59) and did not appear to be reactive.

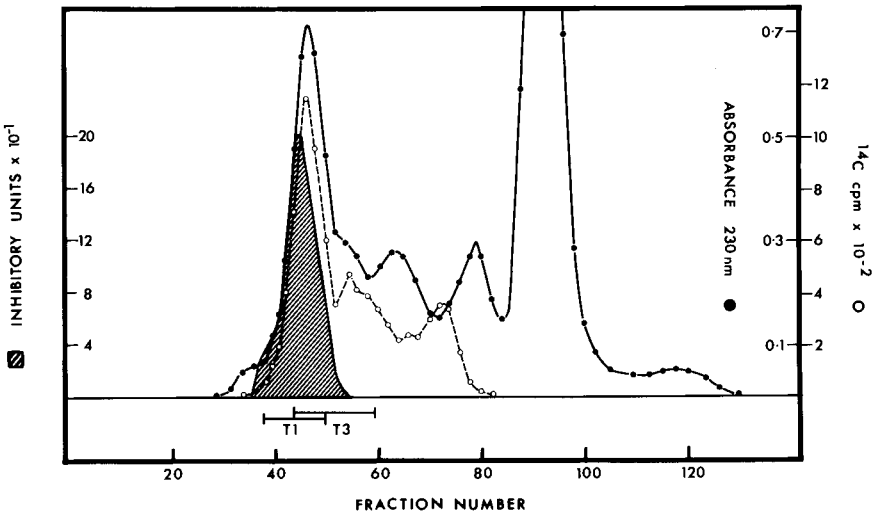

FIGURE 10. Separation of tryptic peptides of Jap CN2.

These two peptides together with the chymotryptic peptide C3 (residues 164-171, carbohydrate at Asn_{164}) and the peptide Th3 (residues 284-289) produced by tryptic and thermolytic cleavage of CN3 were purified by high voltage electrophoresis for final testing. When the isolated glycopeptides were titrated in the inhibition assay (data not shown), only T1 possessed any reactivity with anti-(host antigen) IgG although the level of inhibition obtained was not as great as with the active glycopeptides of X-31 HA_1 at the same concentration.

The carbohydrate compositions of the purified glycopeptides from Jap HA_1 are shown in Table 2. In contrast to Mem

TABLE 2. Carbohydrate Composition of the Oligosaccharide Side-Chains of Jap HA_1

Peptide	Asparagine residue	Sugar composition (moles/mole peptide)			
		N-acetylglucosamine	Mannose	Galactose	Fucose
T1	11	4.7	2.9	3.6	2.1
T3	23	5.2	1.9	4.4	1.3
C3	164	2.5	4.7	1.2	0.4
Th3	285	3.1	3.4	2.6	0.5

and X-31, all oligosaccharide units are of the N-acetyllactosamine type. Direct amino acid sequencing established that the carbohydrate unit on the chymotryptic peptide C3 is attached to the first asparagine residue in the sequence Asn.Asn.Thr.Ser.Gly.Glu.Gln.Homoser, which occurs at position 164 in Jap HA_1. The asparagine residue at position 165 was recovered in good yield and does not carry carbohydrate.

E. Oligosaccharide Side-Chains of HA_2

Both the Asian and Hong Kong HA_2 polypeptides have carbohydrate at asparagine residue 154. These HA_2-associated oligosaccharide units were also found to be antigenically active since anti-(host antigen) IgG was capable of binding to wells coated with purified HA_2 from Mem (H3), X-31 (H3) and Jap (H2) HA. Furthermore, the determinants recognized by anti-(host antigen) IgG on HA_2 are the same as those recognized on HA_1 since the HA_2 chain is capable of complete inhibition in the system with radioiodinated HA_1 as the indicator antigen.

Glycopeptides from X-31 HA_2 prepared for carbohydrate composition studies (18) were also tested in an inhibition system with iodinated X-31 HA_1 (Figure 7). Although glycosylation occurs at only one position (Asn_{154}) on this chain, the tryptic glycopeptide T4 (residues 154-163) was isolated in two different forms (a and b). Only T4a, having the carbohydrate composition: GlcNAc 3.9, Man 3.8, Gal 2.3, Fuc 1.5 (moles/mole peptide) was reactive with anti-(host antigen) IgG. The peptide T4b which had approximately one residue less of each sugar (GlcNAc 3.2, Man 2.9, Gal 1.5, Fuc 0.9) was unreactive.

V. DISCUSSION

The cross-reactive carbohydrate determinants present on the oligosaccharide side-chains attached to Mem (H3), Jap (H2), Bel (HO) and shearwater (Hav6) HA_1s are antigenically similar, perhaps identical. Despite differences in number, composition and location of carbohydrate side-chains in different strains anti-viral serum raised against one of these subtypes reacts with the HA_1 of viruses from each heterologous subtype. The extent of this cross-reactivity

and the overall affinity of interaction are virtually equivalent suggesting that the same population of anti-carbohydrate antibodies is reacting to each strain. This conclusion was further supported by the fact that removal of IgG directed against antigenic carbohydrate common to the immunizing virus and shearwater HA left no residual IgG capable of reacting with the other heterologous HA_1s.

The carbohydrate determinants recognized by antibodies directed against heterologous viruses are the same, or at least completely cross-reactive with, those present on a normal component of the host cell in which the virus was grown (chorioallantoic membrane host antigen). No antigenic carbohydrate determinants were detected in addition to those present on the host antigen, since adsorption of anti-viral IgG with host antigen removed all activity against the heterologous viruses.

The oligosaccharide side-chains containing the determinant common to different influenza subtypes and to the host antigen, have been located in three different strains. In two members of the H3 subtype, A/Memphis/102/72 and X-31, the cross-reactive carbohydrate determinants are present on side-chains attached to the polypeptide backbone of HA_1 at asparagine residues 8 and 22. The other oligosaccharide side-chains of HA_1, at residues 38, 81, 165 and 285 are not antigenic as determined by their lack of interaction with anti-(host antigen) IgG. In contrast to the H3 strains the antigenic carbohydrate on A/Japan/305/57 (H2) HA_1 is confined to a single side-chain, located at residue 11. Since the H2 strains lack the first 10 amino acids found at the N-terminus of HA_1 in the H3 strains, this antigenic side-chain of Jap HA_1 is in a similar location on the polypeptide chain to that at position 22 in the H3 strains.

Only one glycosylation site exists on the HA_2 of Mem, X-31 and Jap HAs (at Asn_{154}) and in all cases the side-chain attached to this site can bind to anti-(host antigen) IgG. The carbohydrate determinants of HA_2 and HA_1 are similar since HA_2 can completely inhibit the binding of anti-(host antigen) IgG to HA_1.

Despite the fact that all of the antigenic side-chains described on both HA_1 and HA_2 of Mem, X-31 and Jap HA share a cross-reactive determinant, the carbohydrate compositions of the chains are not identical (Tables 1 and 2). Four or five N-acetylglucosamine residues and one or two fucose residues are present in each case but the numbers of mannose

and galactose units are variable. Higginbotham et al. (31) have shown that the ability of host antigen to block the inhibition of hemagglutination of purified virus by anti-(host antigen) serum was greatly decreased when galactose and fucose residues were chemically modified or removed from the host antigen. They therefore concluded that these residues were present in the site to which antibody binds. It is apparent that the oligosaccharide side-chains on viral hemagglutinin which lack galactose and fucose (oligomannoside type) do not possess anti-(host antigen) IgG binding capacity but the presence of these residues does not necessarily render them antigenic (as for side-chains at position 38 and 81 in Mem HA_1, 81 in X-31 HA_1 and 23, 164 and 285 in Jap HA_1).

It is known that the oligosaccharide side-chains of influenza hemagglutinin and other glycoproteins are attached to the polypeptide backbone at asparagine residues which occur in the sequence Asn-X-Ser/Thr (13), although in Jap HA not all potential sites are glycosylated (29). From the range of different tripeptide sequences (Table 3) at the oligosaccharide attachment sites in the three strains studied (1,17,18,19) it is interesting to note that five of the eight antigenic side-chains are attached at the sequence Asn-Gly-Thr while the other three, the minor cross-reactive side-chains at Asn_8 in Mem and X-31 HA_1 and at Asn_{11} in Jap HA_1, are attached at the sequence Asn-Ser-Thr. Neither of these sequences are associated with non-antigenic carbohydrate side-chains.

Glysine and serine are the smallest of the amino acids having unchanged polar R groups and are often associated with destabilizing α-helix structure. It is possible that these amino acids, when adajcent to the glycosylated aspargine and followed by a threonine residue, allow the required processing for acquisition of an antigenic determinant to occur without charge or steric hindrance. The HA_1 of fowl plague virus has five potential sites for carbohydrate attachment (32). Two of these, at positions 12 and 123, have the tripeptide sequence Asn-Gly-Thr. It will be interesting to determine whether both of these sites carry antigenic carbohydrate or whether the antigenic oligosaccharides are located solely at the N-terminal region as in the three strains examined here.

Table 3. Oligosaccharide Side-Chain Attachment Sites

	Asn residue	Tripeptide sequence	Carbohydrate type	Reactivity with anti-(host antigen) IgG
Mem HA_1	8	Asn-Ser-Thr	N[a]	+
	22	Asn-Gly-Thr	N	+
	38	Asn-Ala-Thr	N	-
	81	Asn-Glu-Thr	N	-
	165	Asn-Val-Thr	O	-
	285	Asn-Gly-Ser	O	-
Mem HA_2	154	Asn-Gly-Thr	N	+
X-31 HA_1	8	Asn-Ser-Thr	N	+
	22	Asn-Gly-Thr	N	+
	38	Asn-Ala-Thr	O	-
	81	Asn-Glu-Thr	N	-
	165	Asn-Val-Thr	O	-
	285	Asn-Gly-Ser	O	-
X-31 HA_2	154	Asn-Gly-Thr	N	+
Jap HA_1	10	Asn-Asn-Ser	-	
	11	Asn-Ser-Thr	N	+
	23	Asn-Val-Thr	N	-
	139	Asn-Pro-Ser	-	
	164	Asn-Asn-Thr	N	-
	165	Asn-Thr-Ser	-	
	285	Asn-Thr-Thr	N	-
Jap HA_2	154	Asn-Gly-Thr		+

[a]N, N-acetyllactosamine type; O, oligomannoside type
Data from reference 1, 17, 18, and 19.

VI. ACKNOWLEDGEMENTS

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VII. REFERENCES

1. Ward, C.W., and Dopheide, T.A. (1980) in "Structure and Variation in Influenza Virus" (Laver and Air, eds), p.27, Elsevier, Amsterdam.
2. Schulze, I.T. (1975) in "The Influenza Viruses and Influenza" (Kilbourne, ed) p. 53, Academic Press, New York.
3. Wilson, I.A., Skehel, J.J., and Wiley, D.C. (1981). Nature 289, 366.
4. Schwarz, R.T., Rohrschneider, J.M., and Schmidt, M.F.G. (1976). J. Virol. 19, 782.
5. Kaluza, G., Rott, R., and Schwarz. (1980). Virology 102, 286.
6. Rott, R. (1980) in "Structure and Variation in Influenza Virus" (Laver and Air, eds) p. 201, Elsevier, Amsterdam.
7. Wiley, D.C., Wilson, I.A., and Skehel, J.J. (1981). Nature 289, 373.
8. Harboe, A. (1963). Acta. Pathol. Micro. Scand. 57, 317.
9. Laver, W.G., and Webster, R.G. (1966). Virology 30, 104.
10. Lindenmann, J. (1977) in "Virus Infection and the Cell Surface" (Poste and Nicolson, eds), p. 291 Elsevier, Amsterdam.
11. Ward, C.W., and Dopheide, T.A. (1976). FEBS Lett. 65, 365.
12. Keil, W., Klenk, H.-D., and Schwarz, R.T. (1979). J. Virol. 31, 253.
13. Neuberger, A., Gottschalk, A., Marshall, R.D., and Spiro, R.G. (1972) in "The Glycoproteins: Their Composition, Structure and Function." (Gottschalk, ed), p. 450. Elsevier, Amsterdam.
14. Klenk, H.-D., Schwarz, R.T., Schmidt, M.F.G., and Wollert, W. (1978). in "The Influenza Virus Hemagglutinin" (Laver, Bachmayer and Weil, eds), p. 83. Springer-Verlag, Vienna.
15. Schwarz, R.T., Schmidt, M.F.G., Anwer, U., and Klenk, H.-D. (1977). J. Virol. 23, 217.
16. Nakamura, K., and Compans, R.W. (1979). Virology 95, 8.
17. Ward, C.W., Gleeson, P.A., and Dopheide, T.A. (1980). Biochem. J. 189, 649.
18. Ward, C.W., and Dopheide, T.A. (1981). Biochem. J., in press.

19. Basak, S., Pritchard, D.G., Bhown, A.S., and Compans, R.W. (1981). J. Virol. 37, 549.
20. Jackson, D.C., Brown, L.E., White, D.O., Dopheide, T.A., and Ward, C.W. (1979). J. Immunol. 123, 2610.
21. Jackson, D.C., Brown, L.E., and White, D.O. (1981). J. Gen. Virol. 52, 163.
22. Ward, C.W., and Dopheide, T.A. (1980). Virology 103, 37.
23. Dopheide, T.A., and Ward, C.W. (1978). Eur. J. Biochem. 85, 393.
24. Ward, C.W., and Dopheide, T.A. (1979). Virology 95, 107.
25. Ward, C.W., Brown, L.E., Downie, J.C., and Jackson, D.C. (1981). Virology 108, 71.
26. Brown, L.E., Dopheide, T.A., Ward, C.W., White, D.O., and Jackson, D.C. (1980). J. Immunol. 125, 1583.
27. Jackson, D.C., Russell, R.J., Ward, C.W., and Dopheide, T.A. (1978). Virology 89, 199.
28. Gething, M.J., Bye, J., Skehel, J.J., and Waterfield, M.D. (1980). Nature (London) 287, 301.
29. Waterfield, M.D., Gething, M.J., Scrace, G., and Skehel, J.J. (1980) in "Structure and Variation in Influenza Virus" (Laver and Air, eds), p. 11. Elsevier, Amsterdam.
30. Waterfield, M.D., Skehel, J.J., Nakashima, Y., Gurnett, A., and Billham, T. (1978) in "The Influenza Virus Hemagglutinin" (Laver, Boehmayer, and Weil, eds), p. 167. Springer-Verlag, Vienna.
31. Higginbotham, J.D., Schoyen, R., Montensson-Egmund, K., How, M.J., and Harboe, A. (1971). Acta. Pathol. Micro. Scand. B79, 349.
32. Porter, A.G., Barber, A., Carey, N.H., Hallewell, R.A., Threlfall, G., and Emtage, J.A. (1979). Nature 282, 471.

VARIATION OF GLYCOSYLATION SITES IN H_1N_1 STRAINS OF INFLUENZA VIRUS[1]

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ABSTRACT

The glycosylation sites in the HA glycoproteins of a series of H_1N_1 strains of influenza virus were compared by gel filtration chromatography of glycopeptides obtained after pronase digestion and also by mapping of tryptic glycopeptides by a reverse phase high pressure liquid chromatography (RP-HPLC) system. Considerable differences were observed among 1977-78 strains of the H_1N_1 subtype. Furthermore it was observed that strains which are more closely related antigenically are also similar in their tryptic glycopeptide profiles, and strains which are not closely related differ in their RP-HPLC profiles. Our results indicate that RP-HPLC is an effective system for the detection of changes in glycosylation sites.

INTRODUCTION

The hemagglutinin (HA) glycoprotein of influenza A viruses contains a number of oligosaccharide chains linked via N-glycosidic linkages to asparagine residues in a defined sequence of amino acids, Asn-x-Thr (or Ser) (1,2,3). Previous studies in our laboratory (1,4) have shown that the oligosaccharides linked to the HA glycoprotein are of three major types: (1) complex or type I, consisting of glucosamine, mannose, galactose and fucose residues; (2) simple or type II, consisting of several glucosamine and a large number of mannose residues, and (3) hybrid type, which are intermediate between the above two types in composition; i.e., they have a high

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mannose content and also contain galactose, glucosamine, and fucose as distal sugars. Considerable strain dependent differences have been observed in the relative amounts of each type of oligosaccharide chain (5,6). This variation was found to be greater in HA_1 than HA_2, and occurred primarily in the amounts of type II oligosaccharide chains. Type II oligosaccharide chains were found to be much more abundant in both H_1 and H_3 subtypes than in the H_0 and H_2 subtypes. Moreover, within different strains of a single subtype, some variation was also observed (5).

The role of carbohydrates in the HA glycoprotein is not clear. Carbohydrates probably protect the polypeptide backbone from proteolytic degradation and are responsible for the hydrophilic character of the HA molecule. Inhibition of glycosylation by tunicamycin, 2-deoxyglucose or glucosamine increases the susceptibility of glycoproteins to cellular proteases, resulting in intracellular degradation (7,8,9). Recent X-ray crystallographic studies have shown that the carbohydrates are all exposed on the outside of the HA trimer and indeed protect several trypsin and chymotrypsin sites (10).

Little information is available on the possible role of carbohydrates in the antigenic variation of the HA molecule. We have been investigating the variation in oligosaccharides linked to the HA glycoproteins of the H_1N_1 serotype. We initially characterized the glycosylation sites of A/USSR/90/77, the prototype 1977 H_1N_1 strain of influenza virus, after separating the glycosylated tryptic peptides from the HA glycoprotein by a combination of ion exchange chromatography and gel filtration (4). The development in our laboratory of a highly sensitive procedure for mapping of glycosylated tryptic peptides by reverse phase high performance liquid chromatography (HPLC) has enabled us to compare the tryptic glycopeptides of closely related strains of influenza viruses (11). We have selected some of the H_1N_1 field strains isolated during 1977-78, which differ in reactivity with monoclonal antibodies and ferret sera (12). In this paper, we describe our results on the comparative analysis of glycosylation sites in the different H_1N_1 isolates and the possible relationship of these differences with antigenic variation.

MATERIALS AND METHODS

Viruses and cells. The following influenza virus strains were used: A/WSN (H_0N_1), A/PR/8/34 (H_0N_1), A/FM/1/47 (H_1N_1), A/FW/1/50 (H_1N_1), A/USSR/90/77 (H_1N_1), A/California/10/78 (H_1N_1), A/Lackland/AFB/3/78 (H_1N_1), A/Fukushima/103/78 (H_1N_1), A/Arizona/14/78 (H_1N_1), A/Brazil/11/78 (H_1N_1) and A/Lackland/7/78 (H_1N_1). The A/USSR/90/77 and A/FW/1/50 strains were obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, MD. All other H_1N_1 strains were obtained from Dr. A. P. Kendal, Center for Disease

Control, Atlanta, GA. Stocks were grown in the allantoic cavity of hen's eggs and used to infect MDCK canine kidney cells for the preparation of radiolabelled virus.

Preparation of tryptic peptides:

Method 1. ^{3}H sugar-labelled influenza virions were purified according to the procedure previously described (13). Purified virions were subjected to mild trypsin treatment in order to remove the neuraminidase polypeptide and to cleave HA to HA_1 and HA_2 (14). HA_1 and HA_2 were then separated and purified by SDS-polyacrylamide gel electrophoresis. Polypeptides were eluted from gels and precipitated with ethanol to remove SDS, and the precipitated glycoproteins were subjected to complete trypsin digestion. Tryptic glycopeptides obtained by the above procedure were separated by a combination of ion exchange chromatography and gel filtration (4).

Method 2: ^{3}H-glucosamine labelled virions were purified and uncleaved HA was isolated by SDS-polyacrylamide gel electrophoresis. Glycoproteins were eluted from gels, precipitated with ethanol and then reduced and alkylated with dithiothreitol and iodoacetamide in 7.5 M guanidine hydrochloride, pH 8.0. The HA polypeptide was then separated from other reagents by desalting on a Bio-Gel P-2 column in the presence of 10% formic acid, and treated with TPCK-treated trypsin (500 ug) in 0.1 M ammonium bicarbonate buffer. The resultant tryptic glycopeptides were then chromatographed by reverse phase HPLC on a Waters C18 u Bondapak column with a phosphoric acid-n-propanol gradient (70:30) according to a procedure described elsewhere (11).

Gel filtration of glycopeptides: Glycopeptides derived by pronase digestion of either purified virions or isolated tryptic glycopeptides were analyzed by gel filtration on a column of Bio-Gel P-6 (1.0 x 115 cm) according to the procedure described previously (14).

Treatment of glycopeptides with endo-β-N-acetylglucosaminidase (endo-H): Tryptic glycopeptides or pronase derived glycopeptides were dissolved in 250 ul of 0.05 M citrate-phosphate buffer (pH 6.5), incubated with endo-H for 24 hrs at 37°, and chromatographed according to the procedure of Robbins et al. (15).

RESULTS

Analysis of glycosylation sites of the HA glycoprotein of A/USSR virus. When ^{3}H-mannose labelled pronase-derived glycopeptides of A/USSR influenza virus were analyzed by gel filtration (Fig. 1A), three distinct peaks were resolved. In order to determine the type of oligosaccharide chains associated with each size class, we studied the effect of endo-H treatment on the glycopeptides (Fig. 1B). The results show that peak III was completely susceptible to the enzyme, and peak I was resistant. Peak II appeared to be at least partially susceptible to endo-H. The endo-H sensitivity of peak III, and its size

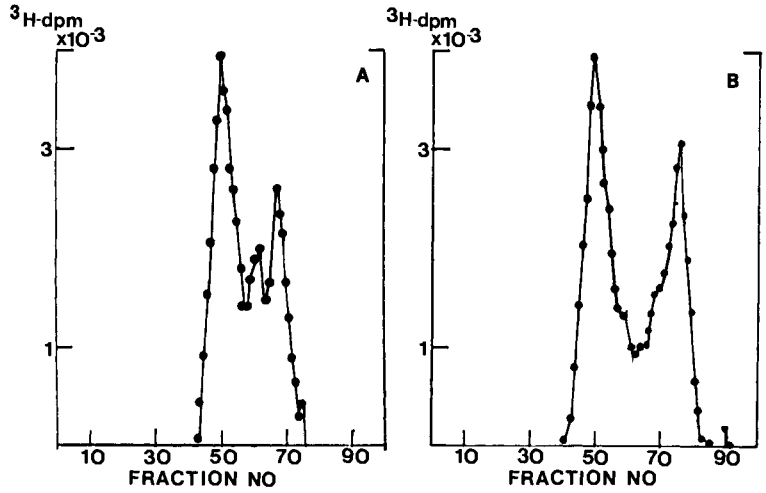

Figure 1. Glycopeptides from A/USSR influenza virus grown in MDCK cells in the presence of ^{3}H-mannose. Purified virions were extensively digested with pronase and the resulting glycopeptides were analyzed by gel filtration on Bio-gel P-6 (A). Fractions 40-78 were pooled, treated with Endo-H and re-chromatographed (B).

as determined from the elution volume, suggest that high mannose type oligosaccharide chains are present. Peak I, on the other hand, apparently contains type I or complex type oligosaccharide chains.

We characterized the glycosylation sites of the A/USSR strain in more detail so that the drift strains appearing during 1978 could be readily compared. Table 1 summarizes the results of analyses of the tryptic glycopeptides of the HA glycoprotein as determined from a combination of ion exchange chromatography, gel filtration, pronase and endo-H digestion, and determination of carbohydrate and amino acid composition (4). Our results suggest that as many as eight classes of tryptic glycopeptides can be present, and that each tryptic fragment possesses a specific type of oligosaccharide chain. From carbohydrate composition analysis using electron capture detection of trifluoroacetate derivatives of the sugar methyl glycosides, and from the susceptibility towards endo-H, we have concluded that 3 major types of oligosaccharide chains are associated with the HA glycoprotein (Table 1) (4,16).

Table 1

Tryptic Glycopeptides of HA_1 and HA_2 Glycoproteins of A/USSR/90/77 Virus

Polypeptide	Tryptic Fragments	Size Before Pronase Digestion	Size After Pronase Digestion	Type of Carbohydrate Chain
HA_1	Ia	3200	3200	Hybrid
	Ib	2700	2500	Hybrid
	Ic	1600	1500	Type II
	II	2900	2700	Hybrid
	III	3100	2800	Type I
	IVa	4000	2800	Hybrid
	IVb	3000	2800	Hybrid
	V	4100	2700	Type I
HA_2	I	2800	2800	Type I

[a]Tryptic glycopeptides were separated by a combination of ion exchange chromatography and gel filtration. Sizes were determined by co-chromatography with ^{14}C-glucosamine labeled pronase digested marker Sindbis glycopeptides. The type of carbohydrate chain associated with each glycopeptide class was determined by monosaccharide analysis by gas chromatography, sensitivity towards Endo-H, and incorporation of different sugar precursors. From Basak et al. (4).

Comparison of glycosylation sites in the HA glycoproteins of different H_oN_1 and H_1N_1 strains of influenza virus. We have examined several other H_oN_1 and H_1N_1 strains in order to determine whether the relative amount of each glycopeptide type varies in the HA glycoproteins (Table 2). The results indicate that A/USSR is similar to the A/FW strain, but considerable differences exist in the relative amounts of oligosaccharide types when other strains are compared. This variation was primarily found to occur in the HA_1 polypeptide. The differences observed could be due either to changes in the number of glycosylation sites or changes in oligosaccharides linked to particular glycosylation sites.

Table 2

Summary of Oligosaccharide Types Associated With HA_1 and HA_2 of Influenza A Viruses Grown in MDCK Cells

Serotype	Virus strain	Oligosaccharide types associated with glycoproteins[a]					
		HA_1			HA_2		
		I[b]	IIa[c]	IIb[d]	I	IIa	IIb
H_oN_1	A/WSN	++	++	-	++	-	-
	A/PR/8/34	++	-	-	++	-	-
H_1N_1	A/USSR/90/77	++	++	++	++	+	-
	A/FW/1/50	++	++	++	++	+	-
	A/FM/1/47	++	+	+	++	-	-

a) The amounts of each glycopeptide type in HA_1 or HA_2 were scored on the basis of elution profiles of ^{3}H-mannose-labeled glycopeptides from isolated glycoproteins. (++) Clearly resolved as a peak. (+) Not clearly resolved but demonstrable as shoulder. (-) Not detected but may be present in trace amount. From Nakamura and Compans (5).
b) Type I glycopeptides.
c) Larger type II glycopeptides.
d) Smaller type II glycopeptides.

To further investigate these differences, we compared the glycosylated tryptic peptides of different H_1N_1 strains. We have developed a method to analyse the tryptic glycopeptides by a reverse phase HPLC system (11). The tryptic glycopeptides of A/USSR were analyzed as described in Materials and Methods, and eight distinct peaks were obtained with a major peak near fraction 30-35 (Fig. 2). Since the HA glycoprotein has been shown to be highly sulfated, we have also analyzed the effect of sulfation on this separation procedure since some peaks could be the sulfated forms of others (14,17). Our result using ^{3}H glucosamine and $^{35}SO_4$ labelled tryptic glycopeptides have shown that peaks 7 and 8 (fractions 66 and 75) are highly sulfated (18). Higher retention of highly charged tryptic peptides in a reverse phase column was surprising, but has been observed in other systems (19) and might reflect formation of

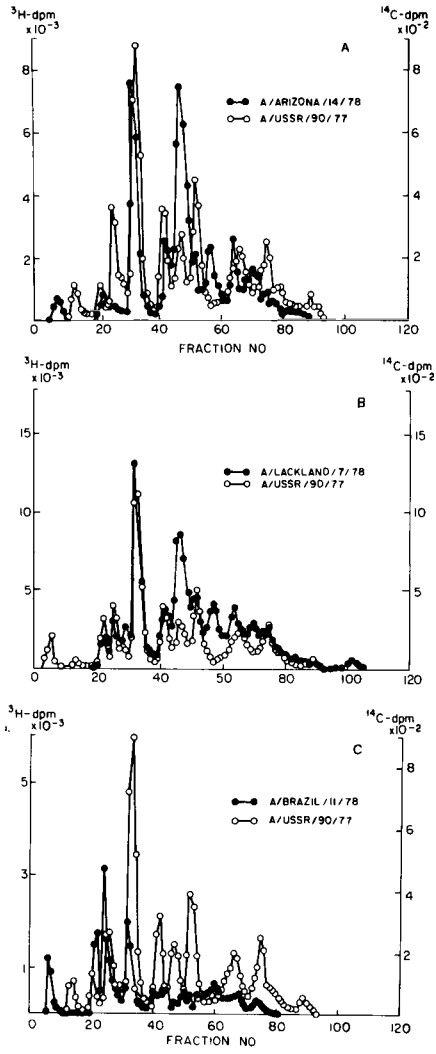

Figure 2. Tryptic glycopeptide profiles of different H_1N_1 isolates analyzed by reverse phase HPLC. ^{3}H-glucosamine labelled virions (A/Arizona/14/78, A/Brazil/11/78 or A/Lackland/7/78) were grown in MDCK cells. HA glycoproteins were purified by SDS-PAGE, mixed with ^{14}C-glucosamine labelled purified HA glycoprotein of A/USSR virus, and tryptic glycopeptides were prepared according to method II as described in Materials and Methods. Co-chromatography of (A) A/Arizona and ^{14}C-glucosamine labelled A/USSR glycopeptides; (B) A/Lackland/7/78 and A/USSR glycopeptides; (C) A/Brazil/11/78 and A/USSR glycopeptides.

aggregates with some other hydrophobic peptides or ionic bond formation.

We compared the tryptic glycopeptide pattern from A/USSR with that of several 1978 H_1N_1 isolates. ^{3}H-glucosamine labelled HA glycoproteins from each strain were isolated, co-digested with ^{14}C-glucosamine labelled HA of A/USSR, and then chromatographed. The results indicated that A/California/10/78 and A/Lackland/AFB/3/78 did not differ to any significant extent from the A/USSR strain (not shown). The tryptic glycopeptide profile of A/Fukushima/103/78 was also very similar to that of the A/USSR strain, except that much higher ratios of some peaks were observed; moreover, some peaks were found to be very heterogeneous (18). The lower isoelectric point reported for the A/Fukushima HA_1 polypeptide by Leavitt et al. (20) might reflect higher sulfation of the glycopeptides resulting in an increase in heterogeneity and relative amount of certain peaks. However, when A/Brazil/11/78, A/Arizona/14/78 and A/Lackland/7/78 were compared with the A/USSR strain, significant differences were observed (Fig. 2). Almost all of the tryptic glycopeptides were observed to have changed. In the A/Arizona strain, peaks 2, 6 and 8 were absent, but there were new peaks at fractions 55-60, 62-64 and 68-72, and another major peak appeared at fractions 45-50. When Lackland/7/78 was compared with A/USSR, peak 5 was found in larger amount and three new peaks appeared at fractions 55-60, 61-65 and 68-74. The A/Brazil/11/78 strain was found to have fewer glycosylated tryptic peptides when compared to the other strains, as indicated by the HPLC profile. Only peaks 1, 2 and 3 were found to be present. The lower molecular weight of the HA glycoprotein of A/Brazil, compared to the other strains studied, also probably reflects fewer glycosylation sites in this strain (18).

In order to investigate the possible relationship of the changes in glycosylation sites with antigenic variation, we have compared the H_1N_1 strains by an HI test using antibody to the A/USSR strain (18). The results showed that those strains which have a similar tryptic glycopeptide map by reverse phase HPLC also reacted similarly to A/USSR in the HI test with A/USSR antibody; strains such as A/Arizona, A/Brazil and A/Lackland/7/78 which differ significantly in their tryptic maps also differed considerably in the HI test. Thus a direct correlation exists between changes in antibody recognition and the changes in the glycosylation profiles. Studies are in progress to determine the exact number of glycosylation sites so that the changes in the RP-HPLC can be correlated with the exact changes in the HA molecule.

DISCUSSION

The variation in the carbohydrate content of the HA glycoproteins of different strains of influenza virus has prompted us to

investigate the role of glycosylation in the antigenic variation of the HA molecule. We have selected the H_1N_1 subtype of influenza virus isolated during 1977-78, since strains of this subtype show more rapid antigenic drift than was observed in the H_3N_2 and H_2N_2 subtypes (12). In order to investigate the changes in the glycosylation sites, we have selected several H_1N_1 isolates of 1977-78 which have been grouped by Webster et al. (12) according to their reaction with different monoclonal antibodies and ferret sera. We have compared the tryptic glycopeptide maps of the HA glycoproteins of these H_1N_1 viruses by a reverse phase HPLC system. Our results show that this procedure can readily distinguish differences in tryptic glycopeptides, although there are some uncertainties in ascertaining the exact numbers of glycosylation sites by this procedure. We have studied the effect of sulfation on the profile obtained by this system (18). Certain peaks have been found to be highly sulfated, and might be highly sulfated forms of the other peaks. In addition, some peaks were found to contain much higher amounts of radioactivity than others, and could contain more than one glycosylated tryptic peptide species. Further analysis of each of the separated peaks is necessary in order to specify the exact number of glycosylation sites. In spite of this reservation, it is apparent that the differences found between A/Arizona/14/78, A/Lackland/7/78, A/Brazil/11/78 and the A/USSR/90/77 strain reflect considerable differences in the glycosyl ation sites, since the profiles of the non-sulfated peaks have markedly changed. Although some changes in the tryptic glycopeptide profiles could be due to changes in the possible trypsin cleavage sites, variation in the number of glycosylated tryptic fragments as observed here suggests that changes are occurring in the number of glycosylation sites in these HA molecules. Our observation of a direct correlation between the changes in the tryptic glycopeptide profile and the differences observed with the HI test using anti-A/USSR antisera suggest that changes in the glycosylation sites are often associated with the appearance of new antigenic drift strains.

Recent X-ray crystallographic studies of the HA molecule have suggested that although carbohydrates are not directly associated with the antigenic sites of the H_3N_2 strain, the oligosaccharides are sometimes found at or near the antigenic sites, and could sterically prevent recognition by antibody (21). The 1977-78 H_1N_1 influenza viruses show more rapid antigenic drift than the H_2N_2 and H_3N_2 viruses, and changes associated with the glycosylation sites may play a role in this variation.

REFERENCES

1. Nakamura, K., Bhown, A. S., and Compans, R. W. (1980). Virology 107, 208.
2. Ward, C. W. and Dopheide, T. A. (1979). Br. Med. Bull. 35, 51.
3. Waterfield, M. D., Espelie, K., Elder, K., and Skehel, J. J. (1979). Br. Med. Bull. 35, 57.
4. Basak, S., Pritchard, D. G., Bhown, A. S. and Compans, R. W. (1981). J. Virol. 37,549.
5. Nakamura, K., and Compans, R. W. (1979). Virology 95,8.
6. Schwarz, R. T., Schmidt, M. F. G., Anwar, U., and Klenk, H.-D. (1977). J. Virol. 23,217.
7. Schwarz, R. T. and Klenk, H. -D. (1974). J. Virol. 14, 1023.
8. Schwarz, R. T., Rohrschneider, J. M. and Schmidt, M. F. G. (1976). J. Virol. 19,782.
9. Nakamura, K., and Compans, R. W. (1978). Virology 84, 303.
10. Wilson, I. A., Skehel, J. J. and Wiley, D. C. (1981). Nature 289, 366.
11. Basak, S., and Compans, R. W. Submitted for publication.
12. Webster, R. G., Kendal, A.P., Gerhard, W. (1979). Virology 96,258.
13. Nakamura, K., and Compans, R. W. (1978). Virology 86,432.
14. Nakamura, K., and Compans, R. W. (1977). Virology 79,381.
15. Robbins, P. W., Hubbard, S. C., Turco, S. J., Wirth, D. F. (1979). Cell 12,893.
16. Pritchard, D. G. and Niedermeier, W. (1978). J. Chromatogr. 152,487.
17. Compans, R. W., and Pinter, A. (1975). Virology 66,151.
18. Basak, S., Bhown, A. S. and Compans, R. W. Manuscript in preparation.
19. Holloway, W. (1981) Personal communication.
20. Leavitt, J. C., Phelan, M. A., Leavitt, A. H., Mayner, R. E. and Ennis, F. A. (1979). Virology 99, 340.
21. Wiley, D. C. and Wilson, I. A., and Skehel, J. J. (1981). Nature 289,373.

STRUCTURE OF THE SPIKE GLYCOPROTEIN OF INFLUENZA C VIRUS[1]

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ABSTRACT

Posttranslational cleavage of the precursor glycoprotein gp88 of influenza C virus results in two subunit glycoproteins, gp65 and gp30, each of which exist in two molecular forms. Influenza C glycoproteins were isolated from purified influenza C virions by selective solubilization with Triton X-100 or octylglucoside. Only preparations obtained with octylglucoside showed hemagglutinating activity. Tryptic peptide analysis of the three species of viral glycoproteins, gp88, gp65 and gp30, revealed that gp30 and gp65 are distinct; when the peaks resolved for the two subunit glycoproteins are superimposed, the pattern corresponding to that of gp88 is obtained. The two molecular forms of gp65 have an identical polypeptide backbone as shown by tryptic peptide analysis. The N-termini of gp88 as well as gp65 were resistant to sequential Edman

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degradation. The gp30 terminal sequence contains a preponderance of hydrophobic residues and shows homology with the corresponding sequence of the HA_2 subunit of influenza A viruses, except for an additional N-terminal glycine residue. The glycoprotein spikes on the surface of influenza C virions were found in regular hexagonal arrays, which appear to involve lateral interaction between the glycoprotein molecules, since the spikes sometimes maintain their arrangement in a network upon release from the viral membrane by limited proteolytic digestion, or upon spontaneous disruption of the viral membrane. In infected cells, closely packed surface projections were observed on crescent shaped outfoldings of the plasma membrane, where virus maturation is occurring. The fact that, in most cases, no nucleocapsids can be seen in such regions suggests that nucleocapsids may not be required to initiate outfolding of the plasma membrane in the budding process of influenza C virions.

INTRODUCTION

Influenza C virions possess a multisegmented, single stranded RNA genome with at least seven segments, ranging from 0.94 to 0.23×10^6 in molecular weight (1-3). The major structural proteins as well as the mode of viral replication of influenza C virus are similar to other members of the orthomyxovirus group (4,5). However, a significant difference exists between influenza C virus and other orthomyxoviruses in the nature and function of the viral glycoproteins. In contrast to influenza A and B viruses, the receptor destroying enzyme (RDE) activity shown by influenza C viruses is not an α-neuraminidase (4,6). In addition, influenza C virions contain only a single glycoprotein gene product (gp88) which is posttranslationally cleaved in permissive host cells to yield two smaller glycoproteins, gp65 and gp30 (7). These smaller glycoproteins resemble HA1 and HA2 of influenza A viruses in that they are linked together by disulfide bonds to form a glycoprotein similar to gp88 (8). The posttranslational cleavage of the viral glycoprotein is a prerequisite for the full biological activity of influenza C virus, a characteristic which is shared by all influenza viruses (9,10).

In order to further characterize the biochemical and biological properties of influenza C virus glycoproteins, we have isolated the glycoproteins using nonionic detergents. The isolated influenza C glycoproteins were used to obtain information on the glycopeptides of the individual glycoproteins and also the amino acid sequence at the N-terminus of the cleavage site. In addition we report some observations on the organization of the spikes on the viral envelope and the plasma membrane of infected cells.

MATERIALS AND METHODS

The methods utilized in the present studies are described in detail elsewhere (7,11,12,13).

RESULTS

Isolation of glycoproteins with nonionic detergents. Nonionic detergents have been used to selectively remove the glycoproteins from various enveloped viruses (12). Solubilization of purified influenza C virions with 2% Triton X-100 resulted in separation of the viral glycoproteins into the supernatant fraction, and the non-glycosylated internal virion components into the pellet fraction. Fig. 1 shows the Triton supernatants of virus preparations grown in chick embryo fibroblast (CEF) and chick kidney (CK) cells, analyzed by polyacrylamide gel electrophoresis under reducing and non-reducing conditions. As previously demonstrated, CEF-grown virus contains only one precursor glycoprotein (gpI) when analyzed under non-reducing conditions, whereas two species of glycoproteins, gpI and gpII can be observed in fully infectious CK cell-grown virions (7). Under reducing conditions, gpI migrates in the position previously designated gp88 (14), and gpII is converted into the two subunits, gp65 and gp30. The gp65 and gp30 subunits are each regularly observed as a doublet band (Fig. 1b).

When the isolated glycoproteins were tested for hemagglutinating activity after butanol precipitation, the binding capacity to red blood cells was no longer detected. However, when octyl-glucoside was used as detergent, the isolated viral glycoproteins showed significant hemagglutinating activity after removal of the detergent by extensive dialysis. This may indicate that the viral lipid is necessary for the glycoprotein in order to obtain a configuration which allows biological activity. However, the isolated glycoprotein preparation did not show elution from chicken erythrocytes after 1 hr at room temperature, as was routinely observed with intact influenza C virions. This might suggest that the isolated glycoproteins lost the capacity to destroy the virus receptors on the red cell membrane.

Analysis of tryptic peptides and glycopeptides of viral glycoproteins. Since the difference in the two bands of the glycoprotein subunits as seen in Fig. 1 could be due to a different extent of glycosylation or to differences in the polypeptide portions, we compared their tryptic peptide patterns by ion exchange chromatography. Fig. 2 shows that virtually identical patterns of peptides were obtained for each band in the doublet of gp65 when the tryptic digests were compared. Therefore, gp65 exists in two forms which contain polypeptides with similar amino acid sequences. When the extent of glycosylation of the two gp65 bands was estimated from the $^{3}H/^{14}C$ ratios in virus preparations doubly labeled with ^{3}H-glucosamine and ^{14}C-amino acids, it was found that the upper band had a ratio of 3.5 compared to 2.6 for the lower band, indicating the presence of additional glucosamine residues in the upper band.

In order to characterize the glycosylation sites of gp88, gp65 and gp30 we have analysed the glycosylated tryptic peptides from

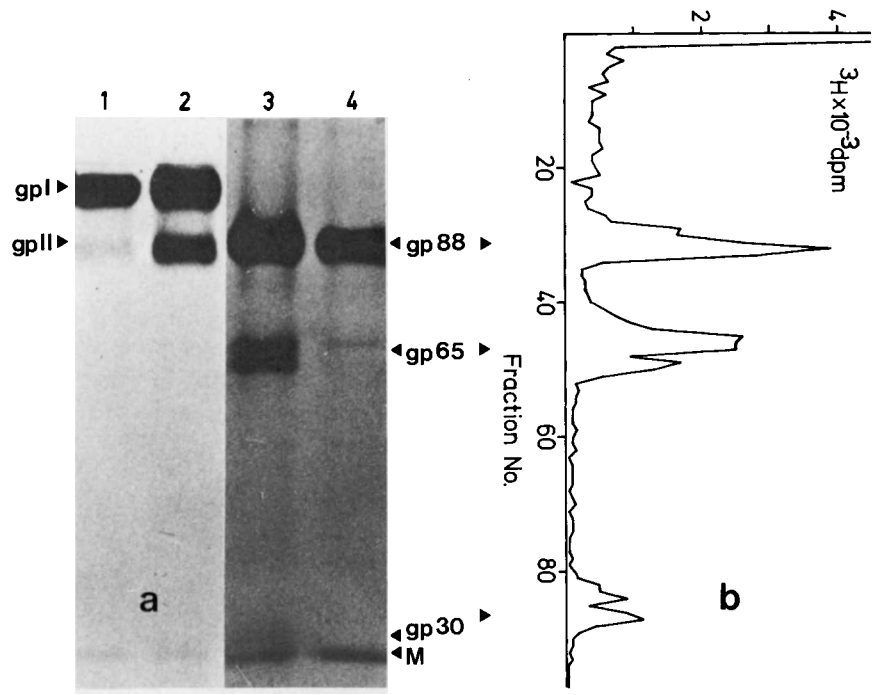

Fig. 1. a. Isolation of the glycoproteins of influenza C virions with Triton X-100. ^{3}H-leucine-labeled influenza C/JHB/1/66 virions grown in CK or in CEF cells were disrupted with 2% Triton X-100 and centrifuged at 100,000 x g for 30 min. The polypeptides of the supernatant were precipitated with n-butanol, analyzed by SDS-polyacrylamide gel electrophoresis and detected by fluorography. Lanes 1 and 4, supernatant fraction, CEF-grown virus; lanes 2 and 3, supernatant fraction, CK grown virus. The samples in lanes 1 and 2 were electrophoresed under non-reducing conditions. Source: Herrler et al (11). b. SDS-polyacrylamide gel electrophoresis of ^{3}H-glucosamine labeled influenza C glycoproteins.

each band by reverse phase high pressure liquid chromatography (Fig. 3). Our results show that the tryptic glycopeptide profiles of gp88 and gp65 are quite similar. As many as 7 different glycosylated tryptic peptide classes were resolved. However, gp30 has fewer glycosylation sites, as expected. The major peaks of gp30 (fractions 22-28 and 65-70) correspond to similar peaks present in gp88. Further analysis will be required to determine whether the number of oligosaccharide side chains is different in the two gp65 bands, or if differences in the composition of the individual oligosaccharide chains is responsible for the difference in electro-

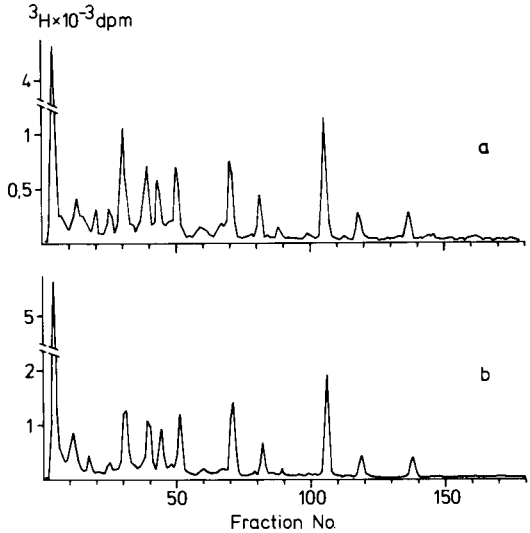

Fig. 2. Tryptic peptide analysis of the doublet band of influenza C gp65 glycoprotein. The virus (JHB/1/66 strain) was grown in CK cells and labeled with ^{3}H-leucine. The upper (a) and the lower (b) band of gp65 were cut from polyacrylamide slab gels, and tryptic peptides were prepared and analyzed as described (11). Source: Herrler et al (11).

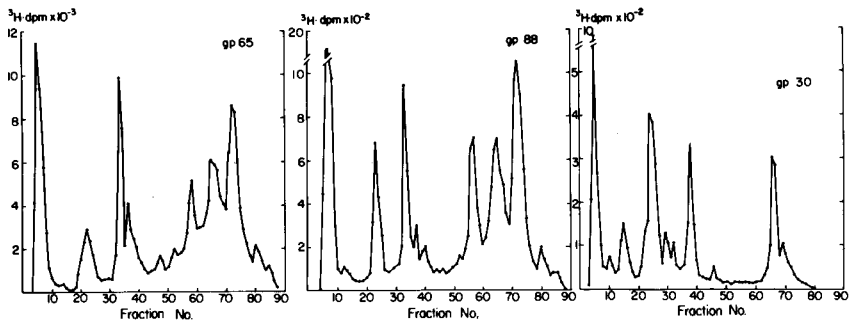

Fig. 3. Tryptic glycopeptide profiles of the influenza C glycoproteins (gp88, gp65, and gp30) analyzed by reverse phase HPLC. ^{3}H-glucosamine labeled virions were grown in CK cells and purified by SDS-PAGE. Sialic acid was removed by neuraminidase treatment and the tryptic glycopeptides were prepared and chromatographed as described elsewhere (13).

phoretic mobility. The possibility also exists that the difference in molecular weight is due in part to differences in the precise sites at which proteolytic cleavage occurred, which might not have been observed in the tryptic peptide patterns.

N-terminal amino acid sequences. To further investigate the structure of the influenza C glycoproteins, we attempted to determine the N-terminal amino acid sequences of gpI and its cleavage products. Also, it was of interest to know whether any homology existed between the sequence observed at the cleavage site of the influenza C glycoprotein, and the highly conserved sequence that has been observed at the N-termini of the HA_2 subunits of the influenza A and B hemagglutinin glycoproteins (15). The gpI, gp65 and gp30 glycoproteins were isolated from SDS-polyacrylamide gels, ethanol precipitated, and 500-1000 picomoles were analyzed by sequential Edman degradation using a liquid phase sequencer. A partial N-terminal sequence was obtained for gp30 (Table I), but it was observed that the N-termini of gpI as well as gp65 were resistant to sequential degradation, suggesting the presence of blocked N-termini. These results suggest that gp65 is derived from the N-terminal portion of the precursor glycoprotein, and gp30 from the C-terminal portion. The gp30 terminal sequence contains a preponderance of hydrophobic residues, and shows homology with the corresponding sequence of the HA_2 subunit of influenza A viruses. In particular, the N-terminal tripeptide sequence Ile-Phe-Gly of influenza C gp30 corresponds exactly to a sequence observed in the HA_2 glycoprotein of A/Victoria/3/75 (H_3N_2) virus, but the latter contains an additional N-terminal glycine residue (16). Such an N-terminal glycine is present on all HA_2 subunits of all influenza A and B viruses that have been analyzed (15). However, a homologous sequence is present at the N-termini of the F_1 subunit of paramyxovirus F glycoproteins, that lacks the terminal glycine residue (17,18), thus resembling the terminal sequence of gp30 of influenza C virus.

Electron microscopic observations on the viral envelope. The glycoproteins on the surfaces of spherical as well as filamentous influenza C virus particles are usually found in regular hexagonal arrays (Fig. 4). The hexagonal organization appears to involve lateral interactions between the glycoprotein molecules themselves, since the spikes are sometimes observed to maintain their arrangement in a network upon release from the viral membrane by limited proteolytic digestion, or upon spontaneous disruption of the viral membrane. When the spikes are viewed end-on, they appear to have a rounded end, as opposed to the triangular shapes of the HA spikes of influenza A viruses in end-on views (19). The influenza C spikes were found to have a length of 8-10 nm and a diameter of 4-5 nm; the center-to-center distance between spikes in hexagonal networks was determined to be 7.5 nm. The glycoproteins isolated by octylglucoside treatment were observed to form rosettes after

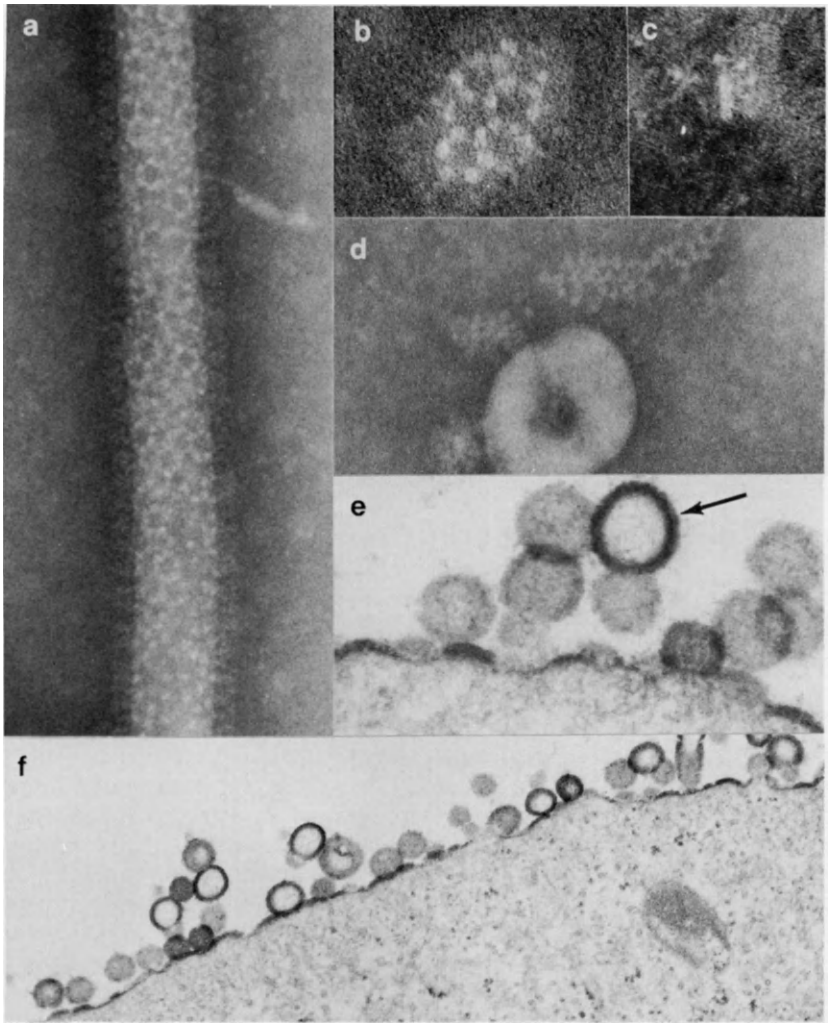

Fig. 4. Electron microscopic observations on the arrangement of influenza C glycoproteins on virions and infected cells. a. Portion of a long filamentous particle with surface projections regularly arranged in a hexagonal pattern. X 215,000. b. Hexagonal arrangement of subunits apparently derived from surface projections of a disrupted virus particle. X 450,000. c. Rosette-like arrangement of glycoproteins isolated with octylglycoside. X 225,000. d. A network of subunits released upon trypsin treatment. X 250,000. e. Thin section of a particle apparently lacking nucleocapsids (arrow), and clusters of projections on the cell surface. X 120,000. f. Low magnification view of surface of an infected MDCK cell, showing many particles which appear devoid of nucleocapsids. X 35,000.

TABLE 1

COMPARISON OF PRIMARY SEQUENCE OF THE AMINO TERMINAL REGION OF gp30 OF INFLUENZA C VIRUS WITH HA_2 OF INFLUENZA A VIRUS AND F_1 OF SENDAI VIRUS

Influenza A Victoria 3/75	NH_2Gly	Ile	Phe	Gly	Ala	Ile	Ala	Gly	Phe	Ile[a]
Influenza C Johannesburg 1/66	NH_2	Ile	Phe	Gly	Ile	?	(Asp)	Leu	Ile	Ile[b]
Sendai virus	NH_2	Phe	Phe	Gly	Ala	Val	Ile	Gly	Phe	Leu[c]

Source: [a]Min Jou et al., (16); [b]Herrler et al. (11); [c]Gething et al., (17).

removal of the detergent by dialysis, as expected for amphipathic integral membrane proteins.

To examine the assembly process of influenza C virions, we examined thin sections of infected MDCK cells, in which the virus undergoes a productive replication cycle (20). In addition to virus particles with typical dense internal cores (Fig. 4), numerous "empty particles" were observed in the process of budding or apparently released. The empty particles possessed an electron dense membrane covered with tightly packed surface projections, enclosing an area apparently devoid of nucleocapsids, with an appearance similar to the cytoplasmic matrix. Frequently, tightly packed clusters of surface projections were also observed on crescent shaped outfoldings of the plasma membrane where virus maturation was occurring; such areas were also apparently devoid of underlying electron dense nucleocapsid structures. These observations suggest that lateral interactions between influenza C glycoproteins may be important in virus assembly, and that nucleocapsids may not be required to initiate outfolding of the plasma membrane for influenza C virus budding.

DISCUSSION

Fractionation with non-ionic detergents results in separation of the influenza C glycoproteins from the internal nonglycosylated components. In contrast to results with other myxoviruses, only glycoproteins isolated by solubilization with octylglucoside showed

hemagglutinating activity, possibly because viral lipids were still present in the glycoprotein preparation. However, since these preparations did not show receptor destroying activity, there is still some uncertainty about the protein responsible for this biological function.

Analysis of tryptic peptide patterns of the doublet bands of the glycoprotein subunit gp65 revealed identical peaks. The cause for the difference in migration of the two molecular forms of gp65 is therefore likely to reside in the sugar moiety of the molecule. So far, the exact difference in glycosylation of the two bands of the viral glycoprotein has not been determined.

Sequence analysis of influenza A and B viruses has shown that the N-terminus of the HA_2 polypeptide consists of hydrophobic residues and is highly conserved. We found that the smallest glycoprotein subunit of influenza C, gp30, has a homologous amino acid sequence at its N-terminus to the HA_2 polypeptide of influenza A viruses, apart from an additional glycine residue which is absent in gp30 of influenza C. Thus, the influenza C gp30 glycoprotein resembles more closely the paramyxovirus F1 glycoprotein, which also lacks an N-terminal glycine residue.

The glycoproteins on intact influenza C virions are arranged in a characteristic hexagonal pattern. Our results indicate that these structures are held together at least in part by lateral interactions between glycoprotein subunits, because they were also found after removal of spikes from the membrane upon protease treatment or after spontaneous release. Influenza C particles devoid of internal nucleocapsid structures, and tightly packed clusters of surface projections were observed on the plasma membrane of infected cells. If lateral interactions occur between the glycoprotein spikes, as suggested by these observations, the hexagonal arrangement would indicate that the spike structure exhibits 3-fold symmetry, as does the influenza A hemagglutinin spike (19).

ACKNOWLEDGMENTS

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REFERENCES

1. Cox, N. J., and Kendal, A. P. (1976). Virology 74, 239-241.
2. Petri, T., Meier-Ewert, H., Crumpton, W. M., and Dimmock, N. J. (1979). Arch. Virol. 61, 239-243.
3. Palese, P., Racaniello, V. R., Desselberger, U., Young, J., and Baez, M. (1980). Phil. Trans. R. Soc. London, B 288, 299-305.
4. Kendal, A. P. (1975). Virology 65, 87-99.

5. Petri, T., Meier-Ewert, H., and Compans, R. W. (1979). J. Virol. 32, 1037-1040.
6. Hirst, G. K. (1950). J. Exp. Med. 91, 177-185.
7. Herrler, G., Compans, R. W., and Meier-Ewert, H. (1979). Virology 99, 49-56.
8. Meier-Ewert, H., Compans, R. W., Bishop, D. H. L., and Herrler, G. (1978). In "Negative Strand Viruses and the Host Cell" (B. W. J. Mahy and R. D. Barry, eds.) pp. 127-133. Academic Press, New York.
9. Klenk, H. -D., Rott, R., Orlich, M., and Blodorn, J. (1975). Virology 68, 426-439.
10. Lazarowitz, S. G., and Choppin, P. W. (1975). Virology 68, 440-454.
11. Herrler, G., Nagele, A., Meier-Ewert, H., Bhown, A. S., and Compans, R. W. Submitted for publication.
12. Scheid, A., Caliguiri, L. A. Compans, R. W., and Choppin, P. W. (1972). Virology 50, 640-652.
13. Basak, S., and Compans, R. W. Submitted for publication.
14. Compans, R. W., Bishop, D. H. L., and Meier-Ewert, H. (1977). J. Virol. 21, 658-665.
15. Skehel, J.J. and Waterfield, M. D. (1975). Proc. Natl. Acad. Sci. U.S.A. 72, 93-97.
16. Min Jou, W. Verhoeyen, M., Devos, R., Saman, E., Fang, R., Hoylebroeck, D., Fiers, W., Threlfall, G., Barber, C., Carey, N., and Emtage, S. (1980). Cell 19, 686-696.
17. Gething, M. J., White, J. M., and Waterfield, M. D. (1978). Proc. Natl. Acad. Sci. U.S.A. 75, 2737-2740.
18. Scheid, A., Graves, M. C., Silver, S. M., and Choppin, P. W. (1978). In "Negative Strand Viruses and the Host Cell" (B. W. J. Mahy and R. D. Barry, eds.) pp. 181-193. Academic Press, London.
19. Wrigley, N. G. (1979). Brit. Med. Bull. 35, 35-38.
20. Nerome, K., and Ishida, M. (1978). J. Gen. Virol. 39, 179-181.

Post-translational Modification of Influenza Virus-induced Proteins During Productive and Abortive Infections

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I. ABSTRACT

The replication of influenza virus is abortive in HeLa cells and the virus yield from these cells is less than 1% of that from permissive cell lines such as MDBK cells. We have previously shown that there is a host cell defect in the final stages of virus maturation which results in a block in the release of influenza virus from HeLa cells and the accumulation of elongated budding virus particles on the plasma membrane (2). In the present study we examined the synthesis and post-translational modification of virus-specific proteins in HeLa cells and MDBK cells infected with influenza virus A/WSN (H1N1) at a multiplicity of 50 PFU/cell. In both cell lines the NP (nucleocapsid protein) and NS_1 (non-structural) polypeptides were detected by 4 hr after infection. In the presence of $^{32}PO_4$, both NP and NS_1 were phosphorylated in MDBK cells; however, only NS_1 was phosphorylated in the abortive infection of HeLa cells. NP was not phosphorylated in infected HeLa cells at any time. The phosphorylation of NS_1 in HeLa cells occurred later and at a reduced level. Virus released from MDBK cells contained phosphorylated NP and the small quantity of virus released from HeLa cells also contained phosphorylated NP. These results suggest that the phosphorylation of NP is important for efficient assembly of influenza virus. The lack of phosphorylation of the NP protein in HeLa cells may alter the charge of the viral ribonucleoprotein (RNP) complexes and modify the interaction of the RNPs with the viral matrix protein (M), resulting in particles of aberrant morphology which fail to bud from the surface of HeLa cells.

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II. INTRODUCTION

Protein phosphorylation is a common post-translational modification observed with many RNA and DNA viruses (7,10,11,13,14,15). The degree of phosphorylation of the vesicular stomatitis virus NS protein has been shown to affect the degree of binding of NS to viral cores (3) and to stimulate transcription in vitro to different degrees (5).

Both the NP and NS_1 proteins of influenza virus are phosphoproteins (8,9). It has been suggested that an endogenous protein kinase activity found in influenza virions may mediate the phosphorylation of the NP protein and thereby stimulate transcription in vitro (4).

The replication of influenza virus is abortive in HeLa cells, resulting in the accumulation of budding virions on the surface of infected cells (2). The assembly of influenza virus appears to require the insertion of the viral glycoproteins into the plasma membrane and the association of the matrix protein (M) with the inner surface of the membrane. The viral ribonucleoprotein complexes, consisting of the RNA genome segments, a major structural polypeptide, NP, and the minor components P_{1-3}, then associate with the regions of the membrane modified by the viral proteins. We have previously reported a reduction in phosphorylation of the viral proteins in abortively-infected HeLa cells (12,13). This study will present evidence that a major function of the phosphorylation of the NP protein is in the assembly of the influenza virion.

III. METHODS

A. Cells and Virus

MDBK and HeLa cells were grown in Reinforced Eagle's Medium (REM) (1) supplemented with 10% newborn bovine serum (Flow Laboratories). Influenza A/WSN/33 (H1N1) was grown in MDBK cells and enumerated by plaque assay in MDCK cells. Virus used for the experiments described had been plaque purified and passaged four times in MDBK cells.

B. General Plan of Experiments

HeLa or MDBK cells were infected with influenza virus at a multiplicity of 50 PFU per cell. The time of addition of virus is designated 0 time. Where indicated, the cells were pulse-labeled with ^{35}S-methionine (20-50 μCi/ml in REM without methionine) for 30 min ending at the indicated time after infection or labeled with $^{32}PO_4$ (50 μCi/ml in REM without phosphate) for 2 hrs ending at the

indicated time after infection. Cumulative labeling was performed similarly; however, unlabeled methionine was included in the culture medium.

C. Immunoprecipitation of Released Virus

Virus released from infected cells was collected after labeling from 2-24 hrs post infection. The culture media were harvested and clarified by centrifugation at 20,000 x g. Human serum containing neutralizing antibody to influenza A/WSN/33 was added to 1% (v/v) and incubated at 37° for 30 min. Goat antiserum to human IgG (Hyland Laboratories) was added to a final concentration of 1% and incubated for an additional 30 min. The immunoprecipitates were collected by centrifugation at 20,000 x g and analyzed by PAGE.

D. Polyacrylamide Gel Electrophoresis (PAGE)

^{35}S- and ^{32}P-labeled proteins were analyzed by PAGE as described by Laemmli (6) using a gradient of acrylamide of 7-15%. The gels were dried and the labeled proteins visualized by autoradiography.

IV. RESULTS

A. Synthesis and Phosphorylation of Influenza Viral Polypeptides During Productive Infection of MDBK Cells

When viral polypeptide synthesis was examined during productive infection, the NP and NS_1 proteins constituted the major viral products in permissive cells (Figure 1). These proteins were synthesized in large amounts by 4 hrs post-infection and were followed by synthesis of the matrix (M) protein which appeared by 5-6 hrs after infection. Cellular polypeptide synthesis shows a sharp decline by 6 hrs after infection. In agreement with the results presented by Privalsky and Penhoet (8,9), NP and NS_1 can also be labeled with $^{32}PO_4$ and the degree of phosphorylation was seen to increase with increasing time after infection. Beyond 8 hrs, virus cytopathic effects result in the loss of cells from the monolayers.

B. Synthesis and Phosphorylation of Viral Polypeptides During Abortive Infection of HeLa Cells

When viral polypeptide synthesis is examined in non-permissive HeLa cells, significant differences are seen in the phosphorylation of viral proteins (Figure 2). The viral NP and NS_1 proteins were first detected at 4 hrs post-infection and increased during the 26 hrs of observation. This longer observation is possible due to the reduced

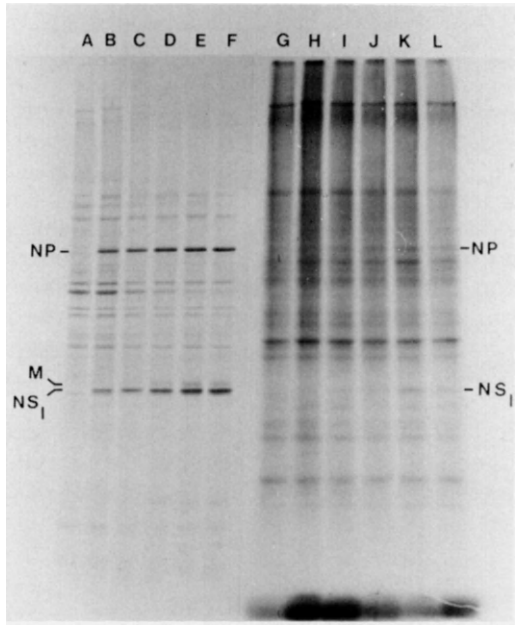

Figure 1. Synthesis and phosphorylation of influenza viral polypeptides during productive infection of MDBK cells. Lanes A-F represent ^{35}S-methionine-labeled samples. Lane A, uninfected cell; Lane B, 4 hrs post-infection; Lane C, 5 hours; Lane D, 6 hrs; Lane E, 7 hrs; Lane F, 8 hrs. Lanes G-L represent ^{32}P-labeled samples. Lane G, uninfected cells; Lane H, 4 hrs post-infection; Lane I, 5 hrs; Lane J, 6 hrs; Lane K, 7 hrs; Lane L, 8 hrs. The position of the viral polypeptides are indicated.

level of cell killing. The most striking difference in the synthesis of viral protein in HeLa cells is in the pattern of phosphorylation of viral proteins. The NS_1 protein appeared to contain a small amount of PO_4 label at 8 hrs and this increased at 18 and 26 hrs. In contrast, the NP polypeptide contained no detectable $^{32}PO_4$ during the time of observation. The observation that the phosphorylation of viral protein is absent or delayed in HeLa cells suggests that the phosphorylation of viral proteins is host cell-dependent and may be related to the defect in maturation of the virus particle (2). It is possible, however, that a significant amount of phosphorylation takes place, but at a slower rate which is not detectable during a 2 hr labeling period. Therefore, viral polypeptide synthesis was examined with cumulative labeling.

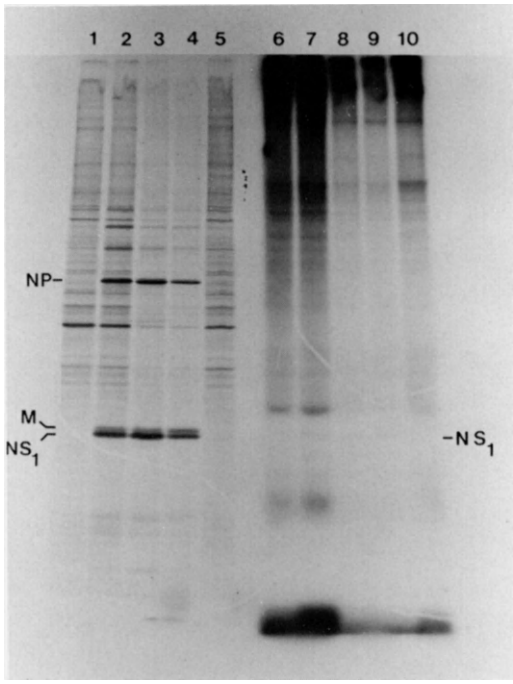

Figure 2. Synthesis and phosphorylation of viral polypeptides during abortive infection of HeLa cells. Lanes 1-5 represent ^{35}S-methionine-labeled samples, and lanes 6-10 represent ^{32}P-labeled samples. Lanes 1 and 6: 4 hrs post-infection; Lanes 2 and 7: 8 hrs; Lanes 3 and 8: 18 hrs; Lanes 4 and 9: 26 hrs; and Lanes 5 and 10, uninfected cells. The positions of the viral polypeptides are indicated.

C. Cumulative Synthesis and Phosphorylation of Viral Proteins During Productive and Abortive Infections

When viral polypeptide phosphorylaton is examined by labeling with $^{32}PO_4$ from 2 to 24 hrs after infection, a different pattern of phosphorylation is seen (Figure 3). In permissive MDBK cells, the only major viral phosphoprotein seen was NS_1. This is to be expected of a non-structural protein which is not released from the cell and accumulates in cytoplasmic inclusions. The phosphorylation of NP is markedly reduced, which would be expected of a structural protein which is exported. A very similar pattern of phosphorylation is seen in HeLa cells with small amounts of $^{32}PO_4$ in NS_1, but none in NP. However, since the HeLa cells also accumulate NP in the form of nascent budding virions, it appears that this NP is not phosphorylated. The HeLa cells do release small quantities of virus (less than 1% as much as MDBK cells), and it is possible that the small quantity of

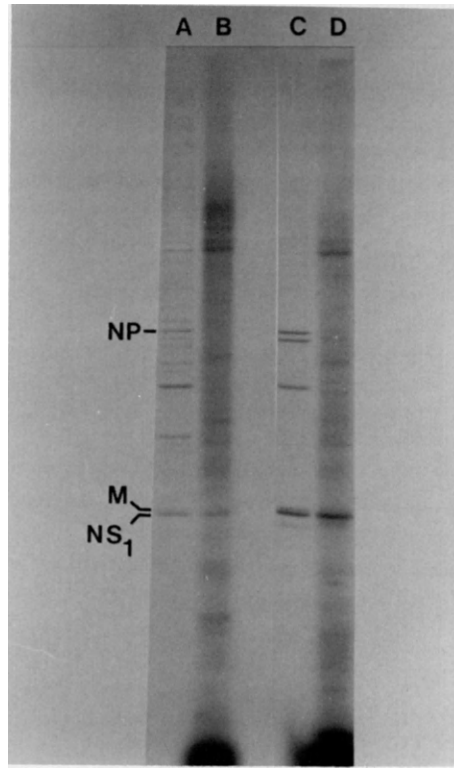

Figure 3. Cumulative synthesis and phosphorylation of viral proteins during productive and abortive infection. The cells were subjected to PAGE and autoradiography as described. Lane A: HeLa cells, ^{35}S-methionine; Lane B: HeLa cells, $^{32}PO_4$; Lane C: MDBK cells, ^{35}S-methionine; Lane D: MDBK cells, $^{32}PO_4$. The positions of the viral proteins are indicated.

virus released has been assembled with phosphorylated NP. Therefore, the virus released from HeLa cells was examined.

D. Phosphorylation of Viral Proteins in Virus Released During Productive and Abortive Infections

Influenza-infected HeLa cells and MDBK cells were labeled with ^{35}S-methionine and $^{32}PO_4$ from 2 to 24 hrs after infection and the virus released from the infected cells was collected from the culture medium by immunoprecipitation with specific antiserum. The immunoprecipitates were examined by PAGE (Figure 4). The virus released from MDBK cells can clearly be seen to contain $^{32}PO_4$ which co-migrates with NP. HeLa cells release considerably less virus, but the viral polypeptides can clearly be seen. While the NP polypeptide is occasionally seen to migrate in two forms, both forms

appear to contain small amounts of $^{32}PO_4$. This suggests that in order for influenza virus to be released from cells, it must contain phosphorylated NP. That protein which accumulates in infected HeLa cells as nascent budding virions is not phosphorylated and not released.

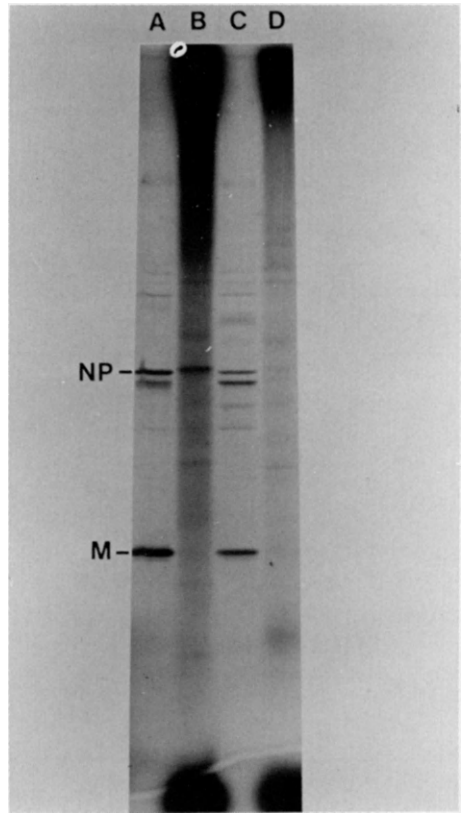

Figure 4. Phosphorylation of viral proteins in virus released during productive and abortive infections. The virus released into the culture medium was collected by immunoprecipitation and examined by PAGE. Lane A: MDBK released virus, ^{35}S-methionine; Lane B: MDBK released virus, $^{32}PO_4$; Lane C: HeLa released virus, ^{35}S-methionine; Lane D: HeLa released virus, $^{32}PO_4$. The positions of the viral proteins are indicated.

E. Estimation of the Degree of Phosphorylation of Viral Polypeptides During Productive and Abortive Infections

The extent of phosphorylation of viral polypeptides was estimated by two techniques as indicated in Table 1. Either the viral bands were excised from polyacrylamide gels and the ^{35}S and ^{32}P content determined by liquid scintillation counting, or the autoradiograms were scanned densitometrically and the area under the peak estimated from the height and width at half-height. It can be seen that the NP from virus released from HeLa cells appears to be under-phosphorylated by 30-40%.

Table I

Estimation of the Degree of Phosphorylation of Viral Polypeptides

Cell Line	^{32}P	^{35}S	$^{32}P/^{35}S$	% Reduction	Estimate*
			NP from Released Virus		
MDBK	59	395	0.149	-	Scintillation Counting
HeLa	11	119	0.092	41	
MDBK	3.1	8.7	0.35	-	Densitometry
HeLa	1.1	4.6	0.24	32	
			NS_1 from Infected Cells		
MDBK	588	113	5.19	-	Densitometry
HeLa	210	61	3.44	34	

* ^{32}P and ^{35}S were estimated as indicated either by liquid scintillation counting of excised bands from polyacrylamide gels or by densitometric scans of autoradiograms of the gels.

When the degree of phosphorylation of NS_1 in infected cells is estimated, a similar reduction in NS_1 phosphorylation is observed in infected HeLa cells.

V. DISCUSSION

The block in replication of influenza virus in HeLa cells has previously been reported to be at a late stage in virus maturation, the final bud formation (2). The synthesis of viral proteins and RNA and the formation and transport of viral ribonucleoprotein complexes appear to be undiminished in abortively-infected HeLa cells when inocula relatively free of defective interfering particles are used (2,13,16). The major defect we have identified in HeLa cells is aberrant phosphorylation of influenza virus proteins. The phosphorylated form of NP is present in the RNPs of the small amount of virus released from HeLa cells; however, the major portion of NP is not phosphorylated and remains associated with the HeLa cells. Although RNPs appear to be assembled in HeLa cells, the RNPs which contain non-phosphorylated NP do not complete the budding process and remain on the cell surface. Thus, our results suggest that the charge difference conferred upon the NP polypeptide is important for the maturation and release of the completed virion. The NS_1 polypeptide is phosphorylated in HeLa cells, but to a lesser extent than that seen in permissive MDBK cells. This is consistent with the observation that the synthesis of viral components occurs normally in HeLa cells (2,13,16). Since the block in the abortive infection is at a late stage in virus maturation and correlates with the phosphorylation of NP, it would appear that the NS_1 is not involved in virus maturation, but rather serves some regulatory role during synthesis of viral components. The significance of the 30-40% under-phosphorylation of NS_1 and of the NP in virions released by HeLa cells is not known, but may reflect differences in the precursor pools of methionine and ATP between the two cell lines.

VI. ACKNOWLEDGEMENTS

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VII. REFERENCES

1. Bablanian, R., Eggers, H.J., and Tamm, I. (1965). Virology 26, 114.
2. Caliguiri, L.A., and Holmes, K.V. (1979). Virology 92, 15.
3. Clinton, G.M., Borge, B.W., and Huang, A.S. (1978). J. Virol. 27, 340.

4. Kamata, T., and Watanabe, Y. (1977). Nature 267, 460.
5. Kingsford, L., and Emerson, S.U. (1980). J. Virol. 33, 2097.
6. Laemmli, U.K. (1970). Nature 227, 680.
7. Pal, B.K., and Roy-Burman, P. (1975). J. Virol. 15, 540.
8. Privalsky, M.L., and Penhoet, E.E. (1977). J. Virol. 24, 401.
9. Privalsky, M.L., and Penhoet, E.E. (1978). Proc. Natl. Acad. Sci. USA 75, 3265.
10. Rosemond, H., and Moss, B. (1973). J. Virol. 11, 961.
11. Russell, W.C., Skehel, J.J., Machado, R., and Pereira, H.G. (1972). Virology 50, 931.
12. Schrom, M., and Caliguiri, L.A. (1981), in "4th International Symposium on Negative Strand Viruses" (Bishop, D.H.L. and Compans, R.W., eds.), Elsevier, New York (in press).
13. Schrom, M. and Caliguiri, L.A. (Submitted for publication).
14. Sokol, F., and Clark, H.F. (1973). Virology 52, 246.
15. Tan, K.B., and Sokol, F. (1972). J. Virol. 10, 985.
16. Caliguiri, L.A. and Gerstein, H. (1978). In "Negative Strand Viruses and the Host Cell" (R.D. Barry and B.W.J. Mahy, eds.) Academic Press, New York, p. 483.

STRUCTURE AND VARIATION OF ANTIGENIC SITES ON INFLUENZA VIRUS HEMAGGLUTININ

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I. ABSTRACT

During antigenic drift in Type A influenza virus, changes in antigenicity are associated with changes in amino acid sequence of the large hemagglutinin polypeptide, HA1. In 10 variants of Hong Kong (H3N2) influenza virus selected with monoclonal antibodies, the proline at position 143 in HA1 changed to serine, threonine, leucine or histidine. In other variants, asparagine 53 changed to lysine, serine 205 to tyrosine, asparagine 133 to lysine, glycine 144 to aspartic acid and serine 145 to lysine. All these changes are possible by single base changes in the RNA except the last, which requires a double base change. Residues 142-146 also changed in field strains of Hong Kong influenza isolated between 1968 and 1977 (1,2).

In order to determine whether sequential changes at the same position occurred during antigenic drift, antibody prepared against the new antigenic site on the variants in which proline 143 changed to histidine or threonine was used to select second generation variants of these variants. In

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the first case, the glycine residue (144) next to the histidine changed to aspartic acid and in the second, the threonine at position 143 reverted to proline and the virus regained the antigenicity of wild-type.

Although monoclonal antibodies revealed dramatic antigenic differences between the variants and wild-type virus, only those variants with changes at position 144 of glycine to aspartic acid or at position 145 of serine to lysine could be distinguished from wild-type virus using heterogeneous rabbit or ferret antisera.

These findings suggest that sequence changes in the region comprising residues 142-146 of HA1 affect an important antigenic site on the H3 hemagglutinin molecule, but how these changes affect the antigenic properties, or whether this region actually forms part of the antigenic site is not yet known. In an attempt to determine which amino acids occur within the antigenic sites, the antigenicity of influenza virus hemagglutinin following chemical modification was examined.

Hemagglutinin molecules from a number of different strains of type A influenza virus were reacted with 1-fluoro-2,4-dinitrobenzene (FDNB), tetra nitromethane (TNM) or diazotized sulphanilic acid (DSA). The substituted molecules had, as far as could be measured, the same capacity as the unsubstituted hemagglutinin to react with heterogeneous antiserum or a panel of monoclonal antibodies.

These results suggest that those amino acid side chains able to react with these reagents (lysine, histidine, tyrosine and cysteine) are either not present in the antigenic sites on the HA, or if they are, then either the side chains which bind antibody do not react with FDNB, TNM or DSA, or the presence of a substituted amino acid in the site does not affect its ability to combine with antibody.

Sequence studies on HA1 from HA molecules treated with FDNB, TNM or DSA showed that certain lysine, tyrosine or histidine residues were 100% substituted after the reaction, while others apparently did not react at all.

II. INTRODUCTION

The hemagglutinin (HA) is one of the surface "spikes" on particles of influenza virus. It is highly immunogenic, inducing antibodies which inhibit hemagglutination and neutralize the infectivity of the virus. However, the hemagglutinin molecule undergoes extensive antigenic variation and antibody induced by infection or vaccination with a particular strain of influenza virus may not be able to neutralize variant viruses. This variation in the structure of the HA is the main reason we are unable to control influenza by vaccination.

Influenza virus HA is a triangular, rod-shaped glycoprotein molecule composed of three pairs of disulphide-linked polypeptide chains, HA1 and HA2 (3). These are coded by one of the 8 single-stranded RNA segments (segment 4) in the influenza virus genome. The complete amino acid sequence of HA1 and HA2 is known for strains within three different subtypes of type A influenza, fowl plague (Hav1N1) virus (4), Asian (H2N2) influenza virus (5) and Hong Kong (H3N2) influenza virus (2,6-9). The N-terminal sequences of HA1 (up to 90 amino acids) has been determined for 32 other virus strains, including representatives of each of the 12 known HA subtypes of type A influenza (Air, in press). Incomplete, bromelain-released HA molecules of Hong Kong (H3N2) influenza virus have been crystallized (10) and the three-dimensional structure has been determined by X-ray diffraction methods (11,12).

The HA of influenza type A viruses undergoes two quite distinct kinds of antigenic variation, antigenic drift and major antigenic shifts. In the latter kind of variation, "new" viruses suddenly appear in the human population with HA (and sometimes NA) molecules totally unrelated antigenically to those of the virus circulating before the new virus appeared. The origin of these new viruses and the way in which they suddenly arise in the human population is not known. They may be viruses which caused epidemics in man many years previously and have remained hidden and unchanged in some unknown place ever since, or they may be derived from animal or avian viruses by mutation or recombination.

Between the major shifts in antigenic structure which define the beginning and end of each pandemic era, the virus undergoes a series of smaller changes. As the human population becomes immune to infection by extant strains of influenza virus, so the pressure rises to select variants

which, by displaying small but significant changes in antigenicity, can evade the immune response. This process is known as antigenic drift. The changes which are responsible for antigenic drift accumulate with time, and field strains isolated several years apart from within a single pandemic era show considerable antigenic differences in their surface antigens (13).

Hemagglutinin molecules from nine strains of A/Hong Kong/68 (H3N2) influenza virus, isolated between 1968 and 1977, were examined for changes in amino acid sequences (1). At least 18 changes, 9 of which were located precisely, occurred in the soluble tryptic peptides of the large hemagglutinin polypeptide (HA1) during this period. These peptides contained 262 residues (82% of HA1). In HA2, only two changes in 129 residues (58% of HA2) were detected.

The nucleotide sequence of the hemagglutinin gene and the deduced amino acid sequence of the protein of the Aichi/68 and Victoria/75 strains have also been compared (2). There were 67 nucleotide differences (3.8%): an insertion in the Victoria gene of three nucleotides close to the amino-terminus of the HA1 (coding for an extra asparagine residue) and 64 nucleotide substitutions. Of these latter changes, 63 occurred in the coding region: 34 were silent nucleotide substitutions and the other 29 changes caused 28 amino acid differences (there is one case, at position 155 of the HA1 of two nucleotide changes resulting in a single amino acid change). Thus, in total there are 29 amino acid differences (including one insertion) or a 5.1% amino acid divergence accumulated over a 7 year period (1968-75).

However, the analysis of amino acid sequence changes in natural influenza variants may not necessarily indicate which portions of the sequences make up the antigenic determinants. Some of the changes observed may be unrelated to the antigenic differences between the various HAs. Consequentjy we do not know which of the changes in sequence found to occur between 1968 and 1977 were responsible for the changes in antigenicity which occurred in the Hong Kong virus during this period.

III. RESULTS

To relate sequence changes to antigenic changes, we have selected antigenic variants of Hong Kong (H3N2) influenza virus under pressure of monoclonal antibodies to different antigenic sites on the HA. The amino acid sequence changes

associated with the altered capacity of the HA to combine with each monoclonal antibody were then determined (14-16).

We found that variants selected with different monoclonal antibodies had totally lost the ability to bind the monoclonal antibody used for their selection (reduction in HI titres were of the order of 50,000 fold). The variants showed single amino acid sequence changes in widely separated regions of the large HA polypeptide, HA1.

These sequence changes (shown in Table 1) were detected by comparing the compositions of the soluble tryptic peptides from the variants with the known sequences of these peptides from wild-type virus. Two insoluble tryptic peptides, comprising residues 110-140 and 230-255 in the HA1 molecule, were not examined and we do not know if additional changes occurred in these regions.

Some variants selected with the same monoclonal antibody showed different sequence changes and some variants selected with different monoclonal antibodies showed the same sequence change. Some monoclonal antibodies to A/Mem/1/71 hemagglutinin were able to discriminate between some of the variants selected with the same monoclonal antibody (Table 2). This may be because a conformational change induced by an amino acid substitution affecting one antigenic site can also affect others, or because some amino acids may be part of two (or more) sites.

This suggests that not all of the antigenic sites on the hemagglutinin are discrete but that considerable overlapping of some sites may occur.

Most of the monoclonal variants could not be distinguished, antigenically, from wild-type virus when heterogeneous antisera (rabbit, mouse or ferret) were used in the tests. Only those variants with changes at position 144 of glycine to aspartic acid or at position 145 of serine to lysine could be distinguished from wild-type virus using heterogeneous rabbit or ferret antisera.

A. Sequential Selection of Variants

When natural variants of Hong Kong virus (field strains) were examined, sequential changes at a particular locus in HA1 were not found. Once an amino acid changed, it did not change again in any subsequent variant examined.

TABLE 1.

Sequence Changes Found in the HA1 Polypeptides from Variants of Type A Influenza Virus is Selected with the Monoclonal Antibodies Listed

Virus	Monoclonal antibody used in the selection	Variant number	Sequence change
A/PR/8/34 (HON1)	PEG-1	V11[a]	(157) Ser → Leu
A/MEM/1/71 (H3N2)	Mem 212/1	V1	(143) Pro → Ser
	Mem 212/1	V2	(143) Pro → Thr
	Mem 212/1	V3	(143) Pro → Leu
	Mem 212/1	V7	(143) Pro → His
	Mem 27/2	V5	(143) Pro → Ser
	Mem 27/2	V9	(143) Pro → Thr
	Mem 123/4	V1	(143) Pro → His
	Mem 123/4	V3	(143) Pro → His
	Mem 123/4	V10	(144) Gly → Asp
	H14/A20	V1	(133) Asn → Lys
	H14/A20	V2	(143) Pro → Ser
	H14/A20	V3	(143) Pro → Leu
	H14/A2	V1	(53) Asn → Lys
	H14/A2	V2	(53) Asn → Lys
	H14/A2	V3	(53) Asn → Lys
	H14/A2	V4	(53) Asn → Lys
	H14/A21	V1	(205) Ser → Tyr
	H14/A20[b]	V1	(145) Ser → Lys
	HK 30/2	V12	(c) Gln → His

(a) 9 other variants selected with PEG-1 probably had the same sequence change (Reference 23 and Brownlee, personal communication)

(b) The third selection in a triple-step selection. The first selection was with H14/A2 and the second with H14/A21.

(c) Between residues 158 and 201 in HA1. Exact position unknown.

TABLE 2

Cross-reactions of the Antigenic Variants with a Panel of Monoclonal Antibodies to A/Mem/1/71 Hemagglutinin

Monoclonal antibody preparation	Wild-type	HI titers with the following variants[a]					
		Selected with H14/A20			Selected with H14/A21		
		V1	V2	V3	V1	V2	V3
Mem 93/1	4.1	<[b]	<	<	+[c]	+	+
Mem 27/2	5.0	5.0	<	<	+	+	+
Mem 212/1	5.1	5.1	2.6	<	+	+	+
Mem 123/4	4.6	4.6	2.5	3.6	+	+	+
H14/B18	4.0	+	+	+	2.5	<	2.5
Mem 200/2	5.0	+	+	+	4.4	3.2	4.4
HK 30/2	4.8	+	+	+	4.4	4.4	4.4
12 other monoclonal antibody preparations	+	+	+	+	+	+	+

[a] HI titers expressed as $\log_{10}$.

[b] Less than 1.7.

[c] Titers for wild-type and variants were identical.

It has been proposed (17-21) that the hemagglutinin molecules of influenza viruses possess a single antigenic site and that antigenic drift occurs by the sequential substitution of increasingly bulky hydrophobic amino acids at a unique locus. However, no direct experimental evidence for this theory has ever been presented.

In order to determine whether sequential changes at the same position occurred during antigenic drift, antibody was prepared against the new antigenic site on the variants in which proline 143 changed to histidine or threonine.

Since the Hong Kong HA molecule appears to possess a large number of overlapping antigenic sites (15) it was thought it would be difficult to prepare monoclonal hybridoma antibodies which bound specifically to the new site. Hyper-immune antisera to the variant HA molecules were therefore absorbed with purified concentrated wild-type virus until the hemagglutinin-inhibition titers of the sera for the wild-type virus reached undetectable levels. The absorbed sera were then tested in HI tests, against the variant viruses.

High levels of HI activity to the variants remained after all HI activity to the wild-type virus had been removed. This antibody to the variants was not removed after repeated absorption of the sera with wild-type virus and therefore must have been directed against a single new antigenic site on the variant HA. In this respect it behaved like "monoclonal" antibody (16).

This absorbed antiserum was used to select second generation variants of the variants in which proline (143) had changed to histidine or threonine. In the first case, the glycine residue (144) next to the histidine changed to aspartic acid and in the second, the threonine at position 143 reverted to proline and the virus regained the antigenicity of wild type (16).

The latter experiment provides the best indication that the monoclonal variants have only single changes in the amino sequence of the HA. Had more than one sequence change been involved in the alteration of antigenic reactivity in the first selection, reversions at the other (unknown) positions must also have occurred in the second selection, which seems unlikely.

The amino acids which changed during the selection of variants with the monoclonal antibodies have been located on the surface of the molecule (12). The areas in which these

changes occurred have tentatively been identified as antibody binding sites (four such sites appear to exist), but it is not certain whether the variable residues are "contact" amino acids or whether, on changing, they induce conformational changes in the rest of the HA molecule and so change the true "binding site" which may be located elsewhere. We have tried to answer this question by examining the effects of chemical modification of certain amino acid residues on the antigenic properties of the HA (22).

We have examined monoclonal variants of A/Mem/1/71 (H3N2) virus in which the change in sequence associated with the alteration of an antigenic site on the hemagglutinin was to an amino acid capable of reacting with 1-fluoro-2,4-dinitrobenzene (FDNB), tetranitromethane (TMN) or diazotized sulphanilic acid (DSA) to form stable derivatives.

We thought that if the amino acids which changed formed part of an antigenic site and were capable of binding antibody, they and the "new" amino acids should also be accessible to the small organic reagents. Furthermore, if these amino acids did form part of the antigenic site, their chemical modification should interfere with the binding of antibody directed against the new site. The experiments should obviously have been done using monoclonal variants in which lysine, tyrosine or histidine residues changed to other amino acids, but so far such variants of Mem/71 virus have not been found.

DNP-substituted hemagglutinin molecules, isolated from FDNB treated A/Memphis/1/71$_H$-BEL$_N$ (H3N1) virus particles, had up to 58% of lysines substituted with DNP. These molecules, nevertheless, retained hemagglutinin activity and, as far as could be measured, the same capacity as the unsubstituted hemagglutinin to react with heterogeneous antiserum or a panel of monoclonal antibodies.

These results suggest that those amino acid side chains able to react with FDNB (lysine, histidine, tyrosine and cysteine) are either not present in the antigenic sites on the HA, or if they are, then either the side chains which bind antibody do not react with DNP, or the presence of DNP in the site does not affect its ability to combine with antibody.

The results also suggest that substitution of more than half of the lysine in the hemagglutinin molecule does not cause any marked conformational changes, for such changes would be expected to affect the ability of the HA to combine with both cell receptors and antibody molecules.

TABLE 3.

Residue and position in HA1		Extent of reaction with 2,4-dinitro-fluorobenzene (pH 9.1 37° 3-4hr)	tetranitro methane	diazotized sulphanilic acid
Lysine	27	NA[b]		
	50[a]	0		
	53	about 60%		
	92	0		
	140[a]	0		
	145	0		
	156	0		
	176	0		
	259	0		
	264	0		
	292	100%		
	299	0		
	307	100%		
	310	100%		
	315	0		
	326	0		
Tyrosine	2		NA	NA
	98		33%[c]	33%[c]
	100		33%[c]	33%[c]
	105		33%[c]	33%[c]
	161		0	0
	178		0	0
	195[a]		0	0
	205		0	NA
	233		NA	NA
	257		0	0
	302		0	0
	308		100%	0
Histidine	17			NA
	18			NA
	56[a]			0
	143			100%
	183			0
	184			0

Similar findings with TNK-treated HA suggest that tyrosine is not an essential part of any antigenic site on the H3 type HA. HA treated with diazotized sulphanilic acid lost HA activity, but its antigenicity was similar to that of untreated HA when tested with heterogeneous antisera, suggesting that histidine was not present in the antigenic sites.

Sequence studies on HA1 from HA molecules treated with FDNB, TNM or DSA showed that certain lysine, tyrosine or histidine residues were 100% substituted after the reaction, while others apparently did not react at all (Table 3). When HA from a monoclonal variant of A Mem/1/71 (H3N2) virus with a sequence change from wild-type in HA1 of proline (143) to histidine was reacted with diazotized sulphanilic acid, the histidine at position 143 in HA1 reacted completely and the HA lost the ability to bind antibody specific for the new antigenic site on this variant. However, treatment of this variant with FDNB did not lead to substitution of histidine 143. No satisfactory interpretation of these results can be made at present.

Legend to Table 3.

(a) Present only in monoclonal variants.

(b) NA = Not analysed (present in insoluble peptides).

(c) Not known if each reacted 33% or one reacted 100% and the others not at all.

Zero reaction means that the peptide containing the numbered residue was present on the maps apparently unchanged in quantity. Some of these peptides may have reacted to some extent, since the total amount of lysine substituted in HA1 was greater than could be accounted for from the data given in the above Table. If low substitution of many peptides did occur, this would not have been detectable from analyses of the individual peptides.

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V. REFERENCES

1. Laver, W.G., Air, G.M., Dopheide, T.A., and Ward, C.W., *Nature*, 283, 454 (1980).
2. Verhoeyen, M., Fan, R., Min Jou, W., Devos, R., Huylebroeck, D., Saman, E., and Fiers, W., *Nature*, 286, 771 (1980).
3. Wiley, D.C., Skehel, J.J., and Waterfield, M., *Virology*, 79, 446 (1977).
4. Porter, A.G., Barber, C., Carey, N.H., Hallewell, R.A., Threlfall, G., and Emtage, J.S., *Nature*, 282, 471 (1979).
5. Gething, M.J., Bye, J., Skehel, J., and Waterfield, M., *Nature*, 287, 301 (1980).
6. Ward, C.W., and Dopheide, T.A., *in* "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds), p. 27. Elsevier, New York, (1980).
7. Sleigh, M.J., Both, G.W., Brownlee, G.G., Bender, V.J., and Moss, B.A., *in* "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds), p. 69. Elsevier, New York, (1980).
8. Threlfall, G., Barber, C., Carey, N., and Emtage, S., *in* "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds), p. 51. Elsevier, New York, (1980).
9. Min Jou, W., Verhoeyen, M., Devos, R., Saman, E., Fang, R., Huylebroeck, D., Fiers, W., Threlfall, G., Barber, C., Carey, N., and Emtage, S., *Cell*, 19, 683 (1980).
10. Wiley, D.C., and Skehel, J.J., *J. Mol. Biol.* 112, 343 (1977).

11. Wilson, I.A., Skehel, J.J., Wiley, D.C., *Nature*, 289, 366 (1981)
12. Wiley, D.C., Wilson, I.A., and Skehel, J.J., *Nature*, 289, 373 (1981).
13. Pereira, M.S., *Brit. Med. Bull.* 35, 9 (1979).
14. Laver, W.G., Air, G.M., Webster, R.G., Gerhard, W., Ward, C.W., and Dopheide, T.A., *Virology*, 98, 226 (1979).
15. Webster, R.G., and Laver, W.G., *Virology*, 104, 139 (1980).
16. Laver, W.G., Air, G.M., and Webster, R.G., *J. Mol. Biol.* 145, 339 (1981).
17. Fazekas de St. Groth, S., *Bull. W.H.O.* 41, 651 (1969).
18. Fazekas de St. Groth, S., *Arch. Environ. Health*, 21, 293 (1970).
19. Fazekas de St. Groth, S., *in* "Negative Strand Viruses: Proceedings" Vol. 2, (B.W. Mahy and R.D. Barry, eds), p. 741 (1973).
20. Fazekas de St. Groth, S., *Top. Inf. Dis.* 3, 25 (1978).
21. Underwood, P.A., *Inf. Immun.* 27, 397 (1980).
22. Laver, W.G., Air, G.M., and Webster, R.G., *Virology*, in press.
23. Laver, W.G., Gerhard, W., Webster, R.G., Frankel, M.E., and Air, G.M., *Proc. Natl. Acad. Sci. U.S.A.* 76, 1425 (1979).

THE ANTIGENIC TOPOLOGY OF THE HEMAGGLUTININ MOLECULE OF INFLUENZA VIRUS A/PR/8/34

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I. ABSTRACT

Two predominantly "strain-specific" (Sa, Sb) and two predominantly "crossreactive" (Ca, Cb) antigenic sites have been delineated previously on the hemagglutinin (HA) molecule of influenza virus A/PR/8/34 by antigenic analysis of PR8 mutant viruses with anti-HA hybridoma antibodies. In the present analysis we show that antibodies directed against sites Sa and Sb bind equally well in RIA to intact virus and isolated HA and HA1 polypeptides whereas antibodies to site Cb bind better to isolated HA and HA1 than to intact virus. Furthermore, antibodies to sites Sa and Sb exhibit, in the

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average, a more than 10-fold higher hemagglutination-inhibition (HI)potency (HI-units/mg antibody) than antibodies to site Cb. These observations are compatible with the idea that the strain-specific sites Sa and Sb, being well accessible to antibody on the intact virus, are probably located on the tip of the HA molecule whereas the cross-reactive site Cb, being less accessible to antibody on the intact virus, is probably localized somewhere between the tip and the base of the HA molecule. The crossreactive site Ca may occupy an intermediate position between the strain-specific sites (Sa, Sb) and the cross-reactive site Cb.

II. INTRODUCTION

We have recently delineated the antigenicity of the hemagglutinin (HA) molecule of influenza virus A/PR/8/34 (PR8) by comparative antigenic analysis of PR8 mutant viruses (Gerhard et al., 1980b, 1981). Briefly, 70 mutant viruses were selected in vitro from parental PR8 virus in the presence of individual anti-HA hybridoma antibodies. Each of these mutant viruses exhibited, compared to the parental virus, a minor antigenic alteration in the HA molecule which resulted probably in most cases from a change in a single amino acid residue in the HA polypeptide (Laver et al., 1979 a,b). An operational antigenic map of the HA molecule was then constructed based on the observation that individual antigenic alterations reduced the capacity of certain groups of anti-HA hybridoma antibodies to bind in the RIA to the corresponding mutant viruses.

Four major antigenic sites, each composed of a cluster of largely overlapping epitopes,[1] could thus be delineated on the HA molecule of PR8. The definition of these sites was based on the observation that antigenic alterations in any given site had no or only a minor effect on the antigenicity of the other antigenic sites. Thus, each antigenic site seemed to represent a structure of the HA molecule which was able to undergo largely independent antigenic alterations.

1) The term epitope (Jerne, 1974) designates the structure of the antigen with which the combining site of an antibody makes contact. This definition of an epitope does not preclude unique epitopes from overlapping structurally.

Two of these sites have been designated Sa and Sb because they contained, as a result of extensive antigenic drift in nature in the H1N1 virus subtype (World Health Organization, 1980), predominantly PR8 "strain- specific" epitopes. The other two sites, designated Ca and Cb, were composed predominantly of "crossreactive" epitopes (i.e. epitypes (Jerne, 1974) that are common to various epidemic H1N1 virus strains).

The four antigenic sites could be further mapped relative to each other by a few antibodies that seemed to recognize parts of two antigenic sites. For instance, some antibodies delineated epitopes that were modified by antigenic alterations in the Sa and Sb sites, indicating a close linkage between these sites. Similar linkages, though less pronounced than between sites Sa and Sb, were observed between sites Sb and Ca and sites Ca and Cb. These observations suggested the presence of a large contiguous antigenic area on the HA molecule in which the sites Sa, Sb, Ca and Cb represent distinct immunodominant antigenic structures.

This antigenic map is, for obvious reasons, entirely operational. Firstly, it is relative to structurally undefined antigenic alterations in the HA molecules of the mutant viruses that have been used in the construction of the antigenic map. For instance, two structurally non-overlapping epitopes could appear to overlap because a residue change in the HA molecule of a mutant virus induced antigenic alterations in distant structures. On the other hand, epitopes that overlap structurally in an antigenic area which has not been altered in any of the PR8 mutant viruses used in the construction of the antigenic map might incorrectly score as non-overlapping. In this regard it should be noted that the mutant viruses used in the antigenic analysis did certainly not exhaust all possible antigenic alterations that could result from single residue changes in the HA polypeptide of PR8 virus. Furthermore, it is conceivable, that certain antigenic structures of the HA molecule can not be altered without concomitant destruction of the functional integrity of the HA molecule. Second, the epitope map has been constructed on the basis of antigenic alterations that detectably changed the binding capacity of antibodies to the mutant viruses (compared to the parental virus) in the RIA. Yet, the sensitivity of the RIA to detect minor changes in an antibody's binding capacity is not precisely defined and, furthermore, may vary from antibody to antibody, depending on its avidity.

In this article, we present experiments that have provided some insight into the topological characteristics of the antigenic map. Our present notion of the antigenicity of the HA molecule of influenza virus PR8 is then discussed in relation to: a) the recent delineation of the three-dimensional structure of the HA molecule of Hong Kong virus by Wilson, Skehel and Wiley (1981), and b) the extent of antigenic drift that occurred in nature in the HA molecule of the H1 serotype.

III. RESULTS

A. The Reactivity of Antibodies with Intact Virus, Isolated HA Molecules and HA1 Polypeptide

HA molecules were released from purified egg-grown PR8 virus with sodium dodecyl-sulfate (SDS) and were subsequently purified by electrophoresis on cellulose acetate strips (SDS-HA). The isolated HA molecules were then dissolved in saturated guanidine-hydrochloride solution and the heavy (HA1) and light (HA2) polypeptides were separated by centrifugation in a guanidine hydrochloride gradient as previously described (Laver, 1973). SDS or guanidine hydrochloride were removed and the purified proteins diluted in phosphate buffered saline (PBS) to give immunoadsorbents of comparable activity (when tested with affinity purified anti-PR8 antiserum) after adsorption into wells of polyvinyl plates (Gerhard et al., 1980b). Anti-HA hybridoma antibodies were then tested in radioimmunoassay (RIA) for their capacity to bind to these immunoadsorbents. The binding capacity was expressed as fraction (on log 2 basis) of antibody binding in the same assay to an immunoadsorbent consisting of intact virus (5 HAU) adsorbed to the plastic plates.

TABLE I. Reactivity of Anti-HA Hybridoma Antibodies with HA and HA1 Polypeptide

RT	Number of Antibodies	Binding (log 2, mean± SD) in RIA to: Whole Virus	SDS-HA	SDS-HA1	Antigenic Site Formed by:
I	27	6.6	6.6±0.3	6.5±0.4	HA1
IIa	3	6.6	4.9±0.9	5.2±0.2	HA1 and/or
IIb	5	6.6	5.9±0.4	0.5±0.8	HA2
III	21	6.6	7.7±0.5	8.3±0.8	HA1

The hybridomas were produced by fusion of lymphocytes from spleen or mediastinal lymph node cells of 12 BALB/c mice after primary or secondary inoculation with influenza virus by the intravenous, intraperitoneal or intranasal routes (Gerhard et al., 1980a). Hybridoma culture fluids were titrated in the RIA against NP40 disrupted PR8 virus immunoadsorbent (Gerhard et al., 1981). One dilution of hybridoma antibody (in linear range of titration curve) was then tested in the RIA for binding to whole virus, HA and HA1. The cpm observed in the interaction with whole virus were defined as 100% (log 2= 6.61). The cpm observed in the interaction of antibody with HA and HA1 were expressed as fraction (on log 2 basis) of the antibody interaction with whole virus.

Table I shows that the antibodies could be grouped on the basis of their binding capacity to HA1 into 4 reactivity types (RT)[1]. Antibodies of RT I reacted equally well with whole virus, SDS-HA and SDS-HA1. Clearly, the epitopes recognized by these antibodies are formed by the HA1 polypeptide and seem to be equally well accessible to antibody on the intact virion and on the isolated HA1 polypeptide. The former applies also to the antibodies of RT III. However, the increased reactivity of these antibodies with the SDS-HA and SDS-HA1 (compared to the intact virus) suggests

1) Since individual antibodies showed various degrees of increased or decreased binding to HA1 we have chosen arbitrary cutoff points which were $\geq$ 7.4 for increased (RT III) and $\leq$ 5.8 for decreased (RT II) binding.

the corresponding epitopes have become better accessible to antibody upon dissociation of the HA and HA1 from the virion. Although SDS-released HA molecules do reassociate into small aggregates (rosettes) upon removal of SDS, electron microscopic observations have shown that the spacing between HA-spikes is larger in rosettes than on intact virions where they form a dense array of surface projections (Wrigley, 1979). Lastly, a small group of antibodies showed decreased (RT IIa) or virtually no (RT IIb) reactivity with SDS-HA1. This could have resulted from partial denaturation of the corresponding epitopes by SDS and, in the case of antibodies RT IIb, from their complete and irreversible denaturation [2] by guanidine hydrochloride. Alternatively, antibodies of RT IIb might recognize epitopes that are formed by the HA2 polypeptide or, more likely, are dependent on the association of HA1 and HA2. At present, neither of the above possibilities can be excluded since we have not been able to use the highly aggregated (hydrophobic) SDS-HA2 polypeptides in antibody binding assays.

B. The Relationship Between RT and Hemagglutination-Inhibition (HI) Potency of Antibodies

The HI-titer and antibody concentration of hybridoma ascitic fluids were determined in standard HI-test (against 4 agglutinating doses of PR8) and RIA (against 4 HAU of NP40 disrupted PR8), respectively. The HI-potency of each antibody was then computed by dividing HI-units/ml by the antibody concentration (mg/ml). It is clear from Table II that antibodies of RT I exhibit, in the average, a 20-fold higher HI-potency than antibodies of RT III. Furthermore, antibodies that showed no detectable HI-activity at antibody concentrations of one or more mg's/ml, were all of RT III. Antibodies of RT II had, in the average, high HI-potency but, as shown by the large standard deviation (SD), formed a rather heterogeneous sample with respect to HI-potency.

Although we cannot exclude at present that the relationship between RT (I and III) and HI-potency resulted from different average avidities of these two antibody groups

2) Since the actual concentration of HA (on intact virus SDS-HA or SDS-HA1) per immunoadsorbent is not known, it is not possible to make a reliable estimate of the extent of denaturation that may have occurred during purification of HA and HA1.

(e.g., RT I larger than RT III), preliminary avidity measurements performed on few antibodies do not support this possibility (Frankel and Gerhard, 1979). The relationship is compatible, however, with the idea proposed above, that antibodies of RT III (in contrast to those of RT I) recognize epitopes that are not fully accessible to antibody on the intact virion.

TABLE II. Relationship Between RT and HI-Potency

RT	HI- units/mg antibody log 10 (mean ±SD)	(number of antibodies)
I	5.07 ± 0.55	(20)
II	4.71 ± 1.10	(7)
III	3.88 ± 0.79	(17)
	< 2.40	(4)

Ascitic fluids from hybridoma bearing BALB/c mice were titrated for HI activity against 4 agglutinating doses of PR8 (using chicken red blood cells) as described by Fazekas et al. (1969). The concentration of anti-HA antibody in the ascitic fluids was determined by RIA as described by Frankel and Gerhard (1979).

C. The Relationship between RT and Site-Specificity of Antibodies

Table III shows that there exists a clear relationship between the antigenic site to which an antibody is directed and its RT. Thus, most antibodies against sites Sa and Sb are of RT I whereas most antibodies against site Cb are of RT III. Antibodies directed against site Ca exhibit in roughly equal proportion RT I and RT III. Thus, if RT I and RT III are a reflection of the accessibility of the corresponding epitopes to antibody, one may conclude from these observations that the strain-specific antigenic sites Sa and Sb are located on the tip of the HA molecule whereas the crossreactive site Cb is formed by structures of the HA1 polypeptide that are removed from the tip towards the base of the HA molecule. The crossreactive site Ca seems to occupy an intermediate topological position.

Some antibodies (3 of RT III directed against sites Sa and Sb and 2 of RT I directed against site Cb) do not fit into this general topological scheme. It is conceivable that these antibodies have been mapped to the incorrect antigenic site via a mutant virus that exhibits a large (conformational) antigenic alteration or several alterations that extend into or are localized in topologically distinct parts of the HA molecule. Also, the assignment of antibodies to given specificity groups by mutant virus analysis was sometimes ambiguous either because the antibody showed reduced reactivity with only a single member of a given mutant virus group or because the antibody showed reduced binding capacity to members of more than one mutant virus group.

TABLE III. Relationship Between Antibody Specificity (determined by virus mutant analysis) and RT

Antibody Specificity	Number of Antibodies Exhibiting RT: I	II	III
Sa	12	2	2
Sb	6	1	1
Ca	5	2	8
Cb	2	2	12

IV. DISCUSSION

The aim of the present study was to assess the topological relationship among antigenic sites of the HA molecule of PR8 virus which we have previously delineated, yet on a purely operational basis, by comparative antigenic analysis of PR8 mutant viruses. Based on 1) antibody competition studies (that have been described in detail elsewhere (Lubeck and Gerhard, 1981), 2) the presently demonstrated differences in antibody binding capacity to intact virus and isolated HA1 polypeptides and 3) the HI-potencies of the anti-HA antibodies, the following topological picture is emerging:

The strain-specific sites Sa and Sb seem to be located in an area of the HA molecule which is fully accessible to antibody on the intact virus. Antigenic analysis of mutant viruses as well as antibody competition studies are compatible with the idea that these sites are in close proximity. They may represent two immunodominant structures in a contiguous predominantly strain-specific antigenic area. Consistent with the presumed locations of these sites is the finding that antibodies against these sites exhibit, in general, high HI-potency (compare Tables II and III) as well as high virus-neutralizing potency (unpublished observations). The latter observations may explain also why these structures underwent drastic antigenic drift in nature and, as a result, became strain-specific.

The crossreactive antigenic site Cb is not as easily accessible to antibody on the intact virion as the strain-specific sites. This suggests that it is not located on the tip but somewhere on the side of the HA spike. Antibody competition studies have unequivocally proven that part of this site is topologically distinct from the Sa site (Lubeck and Gerhard, 1981). The reduced accessibility of the Cb site would also explain the low average HI-potency (compare Tables II and III) and virus-neutralizing potency (unpublished observations) of anti-Cb antibodies. This, in turn, may explain why relatively little antigenic drift occurred in nature in this site.

The crossreactive site Ca seems to occupy, in several respects an intermediate position between the strain-specific sites Sa and Sb and the crossreactive site Cb. In the antigenic analysis of previously described mutant viruses (Gerhard et al., 1981) and of several new mutant viruses (unpublished observations) the Ca site seems to be linked to the Sb as well as to the Cb site. The same general impression has been obtained from antibody competition studies and is further supported by the present observation that roughly equal proportions of anti-Ca antibodies are of RT I and RT III. It is peculiar, however, that the Ca site of PR8 has undergone in nature even less antigenic drift than the Cb site, despite the indication that parts of this site are easily accessible to antibody on the intact virus and the fact that some anti-Ca antibodies exhibit in vitro very high virus-neutralizing potency (unpublished observations). The low extent of drift in the Ca site does not

result from an inherently low mutability of the corresponding protein structures but, instead, may reflect a low immune response of humans to this site (Gerhard et al., 1981).

Wilson, Skehel and Wiley (1981) have recently resolved the three-dimensional structure of the HA molecule of Hong Kong influenza virus. They have further shown (Wiley et al., 1981) that amino acid residue changes which occurred in epidemic virus strains of the Hong Kong subtype or in mutant viruses selected in vitro with monoclonal antibodies clustered into 4 regions of the molecule. Based on this observation, they postulated the presence of four antigenic sites of which A and B were located on the tip of the molecule, C removed from the tip at the junction of the stem and the globular tip of the molecule and D in the interface between adjacent HA monomers. The sites Sa and Sb on the HA molecule of PR8 may be analogous to the sites A and B on the HA molecule of Hong Kong virus and the site Cb may correspond to the site C of Hong Kong.

REFERENCES

Fazekas de St. Groth, S., Webster, R.G., and Davenport, F. M. (1969). J. Immunol. 103, 1099.

Frankel, M.E., and Gerhard, W. (1979). Molecular Immunol.16, 101.

Gerhard, W., Yewdell, J., Frankel, M.E., Lopes, A.D., and Staudt, L. (1980a). In "Monoclonal Antibodies" (Kennett, R.H., McKearn, T.J., and Bechtol, K.B., eds.), p. 317. Plenum Publishing Corp.

Gerhard, W., Yewdell, J., and Frankel, M.E. (1980b). In "Structure and Variation in Influenza Virus" (Laver, W.G., and Air, G., eds.), p.273. Elsevier North Holland, Inc.

Gerhard, W., Yewdell, J., Webster, R.G., and Frankel, M.E. (1981). Nature. In Press.

Jerne, N.K. (1974). Ann. Immunol. (Inst. Pasteur), 1250, 373.

Laver, W.G., and Webster, R.G. (1973). Virology, 51, 383.

Laver, W.G., Air, G.M., Webster, R.G., Gerhard, W., Ward, C.W., and Dopheide, T.A.A. (1979a). Virology 98, 226.

Laver, W.G., Gerhard, W., Webster, R.G., Frankel, M.E., and Air, G.M. (1979b). Proc. Natl. Acad. Sci. USA. 76, 1425.
Lubeck, M., and Gerhard, W. (1981). Virology. In Press.
Wiley, D.C., Skehel, J.J., and Wilson, I.A. (1981). Nature 289, 373.
Wilson, I.A., Skehel, J.J., and Wiley, D.C. (1981). Nature 289, 366.
World Health Organization. (1980). Bull. Wld. Hlth. Org. 58,, 294.
Wrigley, N.G. (1979). Brit. Med. Bull. 35, 35.

ANTIGENIC DRIFT IN INFLUENZA VIRUSES AND ASSOCIATION OF BIOLOGICAL ACTIVITY WITH THE TOPOGRAPHY OF THE HEMAGGLUTININ MOLECULE

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I. ABSTRACT

Monoclonal antibodies to the different antigenic areas on the hemagglutinin molecule of Mem/1/71 (H3N2) influenza virus were studied in an attempt to correlate biological function with the topography of the molecule. The reaction of the monoclonal antibodies with the parental virus and with A/duck/Ukraine/1/63 (duck/Ukr) were determined in an ELISA binding assay, by hemagglutination inhibition and by neutralization of infectivity. None of the monoclonal antibodies to Mem/1/71 HA inhibited hemagglutination of duck/Ukr virus when tested singly but when monoclonal antibodies to different antigenic areas were tested together, inhibition of HA activity was obtained. Although the antibodies failed to inhibit hemagglutination of duck/Ukr virus, ELISA assays showed that they bound to the HA molecule. Based on the results of the ELISA assays, it was shown that

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antigenic area "A" was different on duck/Ukr, whereas the other two areas ("C" and "D") are shared by both viruses.

Monoclonal antibodies to the different antigenic areas on the hemagglutinin molecule differed in their biological properties. Antibodies to one site inhibit hemagglutination, but neutralize infectivity inefficiently, antibodies to another site neutralize infectivity but fail to inhibit hemagglutination. The results are preliminary but do suggest that there may be two distinguishable regions in the binding site on the HA molecule, one for red blood cell receptors, the other for tissue cell receptors.

Previous studies with monoclonal antibodies have shown that antigenic variants can be selected at a high frequency from influenza virus preparations, but few of these differed from the parental virus when tested with ferret antisera. These viruses would have no epidemiological potential. Antigenic variants of A/Texas/1/77 (H3N2) and B/Hong Kong/1/73 were selected with monoclonal antibodies to the HA of these viruses and, although the frequency of isolation of antigenic variation was 2-3 orders of magnitude below the values previously reported, a much high percentage of the variants were 10-fold different from the parental virus with ferret antiserum. Studies with Mem/1/71 showed that after selection of multiple antigenic mutants in antigenic site "A" "significant" antigenic variants were reproducibly isolated. These results suggest that there is no reason why antigenic mutants should not arise by this mechanism in nature.

II. INTRODUCTION

Antigenic drift in the hemagglutinin and neuraminidase molecule of influenza A viruses is a contributing factor in the continuing occurrence of epidemic influenza. Antigenic drift, which involves gradual changes in the surface antigens of influenza virus, is thought to be the result of the selection by an immune population of mutant virus particles having altered antigenic determinants and therefore possessing a growth advantage in the presence of antibody (1). The selection of antigenic variants can be readily achieved with monoclonal antibodies (2) and permits determination of the frequency of variation (3). The primary sequence of a number of different influenza A viruses has recently been determined (4,5,6). During antigenic drift in influenza

viruses, changes in antigenicity are associated with changes in amino acid sequence of the large hemagglutinin polypeptide, HA1 (7,8).

Studies with antigenic variants selected with monoclonal antibodies and sequence analysis of naturally occurring variants suggest that there are 3 to 4 antigenic areas on the hemagglutinin molecule of influenza A viruses (9,10). In the recent elegant studies on the hemagglutinin molecule of A/Hong Kong/68 influenza virus (11,12), four antigenic sites have been associated with the three dimensional structure of the molecule.

Antigenic variants of A/Memphis/1/71 (H3N2) influenza virus, selected with monoclonal antibodies to Groups I, II and III (10), had changes in amino acid sequence at residue 54; 143 and 205, respectively, of the HA1 polypeptide. Although the change in amino acid sequence occurred adjacent to substitutions found in natural variants of the Hong Kong family, few of the variants could be distinguished from the parental virus with heterogeneous antisera. Of the large number of antigenic variants selected with monoclonal antibodies to the hemagglutinin of A/Mem/1/71 (H3N2), only two with substitutions at residues 144 or 145 could be differentiated from parental virus with heterogeneous antiserum. This suggests that the majority of antigenic variants selected with monoclonal antibodies to Mem/1/71 hemagglutinin are epidemiologically irrelevant (10).

In this paper, we will analyze the biological properties of monoclonal antibodies to the different antigenic areas on the hemagglutinin molecule of Mem/1/71 and attempt to relate these findings to the three dimensional structure of the HA molecule. The results show that monoclonal antibodies differ in their biological properties. Some combine and neutralize infectivity, yet fail to inhibit hemagglutination and these properties correlate with the location of the antigenic determinants on the molecule.

In addition, we will show that antigenic variants of A/Texas/1/77 (H3N2) and B/Hong Kong/1/73, that are significantly different from the parent strain, can easily be selected with monoclonal antibodies to the hemagglutinin molecule. In contrast, antigenic variants of Mem/1/71 that are significantly different from the parent virus can only be selected reproducibly after multiple mutations have occurred in one of the antigenic areas.

III. RESULTS AND DISCUSSION

A. Reactivity of Monoclonal Antibodies to Mem/1/71 Influenza Virus Hemagglutinin with A/Duck/Ukraine/1/63 Influenza Virus

According to the revised nomenclature for influenza A viruses (13), duck/Ukraine/1/63 (duck/Ukr) influenza virus that was previously designated Hav7 has been grouped with the Hong Kong/68 (H3N2) subtype. This was based on antigenic and biochemical relationships between the hemagglutinin molecules of the viruses (14). It was, therefore, somewhat surprising when a panel of monoclonal antibodies to H3 hemagglutinin completely failed to inhibit the hemagglutinin activity of duck/Ukr influenza virus (Table 1).

TABLE 1. Reactivity of Monoclonal Antibodies to H3 Hemagglutinin with Duck/Ukraine/1/63 Influenza Virus

Monoclonal antibody	Group	HI reactivity	
		Mem/1/71 (Intact virus)	Dk/Ukr (Intact virus)
Mem/1/71	Ia	+	-
	IIa	+	-
	b	+	-
	c	+	-
	d	+	-
	e	+	-
	IIIa	+	-
	b	+	-
	c	+	-
	d	+	-
	e	+	-
	f	+	-
	g	+	-
Duck/Ukr/63	?a	-	+

The reactivity patterns were determined in hemagglutination inhibition tests (HI) as described (10).

There are several possible explanations for these findings. (i) That the panel of monoclonal antibodies to Mem/1/71 HA contained no cross-reactive antibodies to the duck/Ukr virus; (ii) that antibodies bind to duck/Ukr but fail to inhibit hemagglutination; (iii) that antibodies to H3 are sterically blocked from combining with the antigenic sites on duck/Ukr; (iv) that a cooperative effect between antibody molecules to different antigenic sites is necessary for biological activity.

To test these possibilities, monoclonal antibodies to H3 hemagglutinin were assayed in an enzyme linked immunoabsorbent test (ELISA) with duck/Ukr and in an HI test with isolated HA from duck/Ukr (Table 2,3). It is apparent that

TABLE 2. Reactivity of Monoclonal Antibodies to H3 Hemagglutinin with Duck/Ukraine/1/63 Influenza Virus

Monoclonal antibodies to Mem/1/71 HA Group	ELISA		HI Reactivity
	Dk/Ukr (Intact)	Dk/Ukr (Isolated HA)	Dk/Ukr (Isolated HA)
Ia	+	+	+
IIa	-	-	-
IIIa	-	-	-
b	±	-	±
c	+	+	+

The grouping of monoclonal antibodies to Mem/1/71 has been described (10) and the ELISA assays were done as described (15).

monoclonal antibodies in Group I can combine with intact duck/Ukr, for high titers were obtained in the ELISA assay, but these antibodies failed to inhibit HA activity. On the other hand, monoclonal antibodies from Group I did inhibit the hemagglutinin activity of isolated HA from duck/Ukr. Monoclonal antibodies in Group II failed to combine with duck/Ukr virus. The results suggest that one or more amino acid changes probably occurred in the antigenic area recognized by these antibodies (in the region of residue 144 of HA1). Monoclonal antibodies in Group III showed two patterns of reactivity, Group IIIa and b antibodies failed to combine or combined to very low titers. Antibodies in Group IIIc combined with intact and isolated HA subunits in the

TABLE 3. Reactivity of H3 Monoclones with Duck/Ukraine/1/63 Influenza Virus

Monoclonal antibodies to Mem HA Group	Hemagglutination Inhibition[a]			
	Mem (Intact)	Mem (HA)	Dk/Ukr (Intact)	Dk/Ukr (HA)
Ia	6,000	12,000	<100	40,000
IIa	~100,000	~100,000	<100	<100
IIIc	100,000	~100,000	<100	~100,000
	ELISA[a]			
Ia	64,000	N.D.	32,000	32,000
IIa	16,000	N.D.	<250	<250
IIIc	256,000	N.D.	128,000	128,000

[a]Hemagglutination inhibition and ELISA assays were done as described (10,15). N.D. = Not determined.

ELISA assay, but in HI assays Group IIIc inhibited the hemagglutinin activity of isolated duck/Ukr HA subunits, but not the intact virus. This information supports the possibility that antibodies in Groups I and IIIc can combine with duck/Ukr but the antibodies cannot block HA activity on intact virus. Since these antibodies can effectively inhibit HA activity of isolated HA from duck/Ukr, it suggests that the affinity of binding is not involved.

B. Cooperative Effects of Monoclonal Antibodies

Monoclonal antibodies to the different antigenic determinants on Mem/1/71 were tested singly, and after mixing, to determine if they would inhibit the hemagglutinin activity of duck/Ukr (Table 4). The results show that mixtures of different antibodies to the same antigenic area failed to inhibit hemagglutination but when antibodies to different antigenic areas were mixed, HI activity was detectable. These results suggest that there must be some interaction between the monoclonal antibodies that may stabilize the combination and effectively block the receptor site on the HA molecule.

TABLE 4. Mixtures of H3 Monoclonal Antibodies will Inhibit the HA Activity of Duck/Ukraine/63

Monoclonal antibody Group	HI Activity	
	Dk/Ukr	Mem/1/71
I	<	6,000
II a,b,c,d,e, Mixed	<	100,000
I + II Mixed	600	100,000
I + III Mixed	3,200	~100,000

The grouping of monoclonal antibodies and the serological tests have been described (10).

Although the cross-reactions between Mem/1/71 and duck/Ukr are below those found with heterogeneous antisera (14) (between 2-and 10-fold different), the mixtures of monoclonal antibodies do suggest that cooperative effects may be important in determining the biological outcome of virus antibody interaction.

C. Neutralization of Infectivity in the Absence of HI Activity

The most important aspect of virus-antibody interaction is whether the antibodies will neutralize the infectivity of the virus. Monoclonal antibodies to the different antigenic areas were tested for their ability to neutralize the homologous and heterologous virus (Table 5). Monoclonal antibodies in Group I were inefficient at neutralizing the infectivity of Mem/1/71 and failed to neutralize duck/Ukr. The antibody in Group II neutralized the homologous virus but failed to neutralize duck/Ukr. The antibodies in Group III neutralized the homologous virus to high titers and also neutralized duck/Ukr. When the antibodies from the different antigenic areas were combined, there was cooperation in that higher titers were obtained than when any of the antibodies were tested singly. The finding that antibodies can neutralize infectivity of the virus without inhibiting hemagglutination is unusual and has not previously been reported. These findings could be explained by the greater

TABLE 5. Neutralization of Infectivity of Mem/1/71 and duck/Ukr with H3 Monoclonal Antibodies

Monoclonal antibody	Neutralization titer	
	duck/Ukraine	Mem/1/71
Sendai HN	<30	<30
Mem HA Gp I (a)	<30	80
Gp II (a)	<30	2,000
Gp III (c)	4,000	50,000
Gp I, II, III	18,000	50,000

Neutralization of infectivity was done in chick embryos as described (14).

sensitivity of the neutralization test but this is unlikely, for antibodies in Group III can efficiently inhibit hemagglutination of the isolated HA subunits from duck/Ukr virus. A more likely explanation is that the topography of the HA molecule in the intact virus does not permit the antibody molecule to interfere with the red cell binding site.

D. Association of Biological Activity of Mem/1/71 Monoclonal Antibodies with the Topography of the HA Molecule

Monoclonal antibodies in Group I have been associated with changes in sequence at residue 54 of the HA1 polypeptide and correspond with the antigenic region designated "C" (12). Similarly, monoclonal antibodies in Group II correspond with antigenic region "A", and Group III with "D" (Table 6). No monoclonal antibodies in the Mem/1/71 panel correspond with the antigenic area designated "B", it is possible that antibodies to regions "B" and "D" have been put together in Group III. Monoclonal antibodies to different antigenic areas on Mem/1/71 (H3N2) influenza virus were used to analyze duck/Ukr/1/63 virus. Based on the results of the ELISA assays, it is apparent that antigenic area "A" is different on Dk/Ukr, whereas the other two areas "C" and "D", are shared by both viruses. Antibodies to the "C" and "D" regions behave differently in biological assays on the two viruses suggesting that there must be conformational differences between the two HA molecules.

TABLE 6. Correspondence Between Monoclonal Antibody Groups and Proposed Antigenic Sites on H3 Influenza Virus Hemagglutinin

Mem/1/71 HA Monoclonal antibody Group	Change in amino acid sequence of HA1 in variants	Proposed antigenic site (Wiley et al., 12)
I	54	C
II	143 144 145	A
III	? 148-200	?B
	205	D

The monoclonal antibodies to the antigenic sites "C" and "D" differ in their abilities to inhibit binding of the HA molecule to red blood cells and to tissue cells. Antibodies to region "C" inhibit HA activity of Mem/1/71, but are very inefficient at neutralizing infectivity of this virus. Region "C" is located beneath the head of the HA molecule and antibodies bound to this site apparently influence binding to red blood cells but not to tissue cells. In contrast, antibodies to antigenic region "D" located in the cleavage between the 3 globular heads of the HA molecule, effectively inhibits HA activity and neutralizes infectivity of Mem/1/71. However, antibodies to this antigenic area, when tested on duck/Ukr, failed to inhibit HA activity but effectively neutralized infectivity. This suggests that antibodies bound to this site on duck/Ukr influences binding to tissue cells but not to red blood cells. These results are preliminary but do suggest that there may be two distinguishable regions in the binding site on the HA molecule, one for red blood cell receptors, the other for tissue cell receptors.

E. Selection of "Significant" Antigenic Variants of Influenza Viruses with Monoclonal Antibodies to the HA Molecule

Previous studies (3,10) with monoclonal antibodies to A/PR/8/34 (HON1) and A/Mem/1/71 (H3N2) have shown that antigenic variants can be selected with a high frequency; approximately 1 in 100,000 particles are antigenic variants. Analysis of these antigenic variants with post infection ferret sera has shown that few of the variants could be distinguished from the parental virus. The great majority of the variants would, therefore, have no epidemiological advantage since a "significant" antigenic variant is defined as one that is at least four-fold different from the parental virus in HI tests with ferret antiserum.

Studies were, therefore, done with monoclonal antibodies to the HA molecule of A/Texas/1/77 (H3N2) and with B/Hong Kong/1/73 (16) to determine if these viruses also showed a similar frequency of antigenic variation and whether more of the variants would have epidemiological potential (Tables 7 and 8). Selection of antigenic variants with

TABLE 7. Selection of "Significant" Antigenic Variants of A/Texas/1/77 with Monoclonal Antibodies to the HA Molecule

Monoclonal Antibody Number	Frequency of variants ($\log_{10}$)	Difference from parent virus with ferret serum in HI test (fold)
10/1	< -7.40	-10
39/4	-3.82	0
26/2	< -7.40	-10
29/1	< -7.40	- 5
52/1	-6.20	- 4
77/3	< -7.40	0

The selection and analysis of antigenic variants were done as described (10).

monoclonal antibodies to different antigenic areas on A/Texas/1/77 gave an overall low frequency of antigenic variants. The frequency of variation was at least two orders of magnitude below the average frequency ($10^{-5.0}$) with Mem/1/71 and PR/8/34. On the other hand, antigenic variants

TABLE 8. Selection of "Significant" Antigenic Variants of B/Hong Kong/1/73 with Monoclonal Antibodies to the HA Molecule

Monoclonal Antibody		Frequency of variants (log_{10})	Difference from parent virus with ferret serum in HI test (fold)
Group	Number		
Ia	16/1	-8.05	+ 4
	313/2	-7.25	+ 2
	430/1	< -7.20	0
Ib	282/1	-7.84	+ 2
	365/1	-7.84	-20
Ic	21/6	-7.38	-20

The selection and analysis of antigenic variants were done as described (16).

selected with 4 of the 6 monoclonal antibody preparations were from 4- to 10-fold different from the parental virus in HI tests with ferret antiserum. Similar results were obtained with the B/Hong/Kong/1/73 influenza virus; antigenic variants were isolated at low frequency but the variants selected with monoclonal antibodies in Groups Ib and Ic were 20-fold different from the parental virus (Table 8).

These results raise the question of why only two "significant" antigenic variants of A/Mem/1/71 were selected in previous experiments either after direct selection or after multiple steps of selection with antibodies to different antigenic areas (10). In an attempt to resolve this question, further studies were done with monoclonal antibodies to different overlapping regions of a single antigenic site before variants were selected with antibodies to another antigenic area.

The results (Table 9) show that, after three sequential passages in different monoclonal antibodies to Group II, the antigenic variants failed to react with any monoclonal antibodies in this group; nevertheless, the variant (V3) could not be differentiated from parental virus with ferret antiserum. When antigenic variants were selected from this virus preparation (V3) with monoclonal antibodies belonging to Group III, antigenic variants different from the parent

TABLE 9. Sequential Selection of "Significant" Antigenic Variants of Mem/1/71

Virus	Variant selected with	Reactivity patterns in HI assays with monoclonal antibodies to the following groups						Fold difference from parent with ferret antisera in HI test
		Group II				Group III	Group I	
		a	b	c	d	a-e		
Mem/1/71		+	+	+	+	+	+	0
V1	IIa	-	+	+	+	+	+	0
V2	IIb	-	-	-	+	+	+	0
V3	IId	-	-	-	-	-	+	0
V4 (a)	IIIa	-	-	-	-	-	+	-10
V4 (b)	IIIb	-	-	-	-	-	+	-10
V4 (c)	IIIc	-	-	-	-	-	+	- 8

The selection and analysis of antigenic variants were done as described (10).

virus with ferret sera were reproducibly isolated. This was the case whether antibodies IIIa, IIIb or IIIc were employed in selection. These studies show that it is possible to reproducibly isolate "significant" antigenic variants from Mem/1/71 influenza virus with monoclonal antibodies.

The difference in amino acid sequence between the Mem/1/71 parent and the variants V1, V2, and V3, has not been determined but, in all likelihood, the substitutions occur in antigenic area "A" that comprises an external loop on the HA molecule. We do not know why V3 can reproducibly give mutants that are "significantly" different but it can be postulated that the conformation of the molecule is readily altered by subsequent mutations.

Among the natural variants of Hong Kong/68 (H3N2) influenza viruses that have been sequenced, amino acid substitutions were first detected at residue 144 and in later strains at other sites (7). In nature, antigenic variants are infrequently encountered early after introduction of a new subtype and more frequently isolated at later times, suggesting that as more mutations accumulate in the HA molecule that it becomes more prone to conformational changes. Both A/Texas/1/77 (H3N2) and B/Hong Kong/1/73 are "senior" variants and might be expected to have acquired many mutations. The low frequency of isolation of antigenic variants in these strains suggest that many of the mutations are probably lethal.

The above studies do establish that "significant" antigenic variants of influenza viruses can be selected with monoclonal antibodies, and that the frequency of isolation was low (approx. 10^{-7}). Whether this is the method of selecting antigenic variants in nature is unknown but since a single mutation can result in a virus that is antigenically "significantly" different, there seems to be no reason why this should not occur in nature.

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V. REFERENCES

1. Francis, T., Jr., and Maassab, H.F., in "Viral and Rickettsial Infections of Man" (F.L. Horsfall and L. Tamm, eds.), 689. J.B. Lippincott Co., Philadelphia, Penn., (1965).
2. Gerhard, W., and Webster, R.G., J. Exp. Med. 148, 383, (1978).
3. Yewdell, J.W., Webster, R.G., and Gerhard, W., Nature (Lond) 279, 246, (1979).
4. Porter, A.G., Barber, C., Carey, H.N., Hallewell, R.A., Threlfall, G., and Emtage, J.S., Nature 282, 471, (1979).
5. Ward, C.W., and Dopheide, T.A., in "Structure and Variation in Influenza Virus" (W.G. Laver and G. Air, eds.), 21, Elsevier, North Holland, (1980).
6. Min Jou, W., Verhoeyen, M., Devos, R., Saman, E., Fang, R., Huylebroeck, D., and Fiers, W., Cell 19, 683, (1980).
7. Laver, W.G., Air, G.M., Dopheide, T.A., and Ward, C.W., Nature 283, 454, (1980).
8. Verhoeyen, M., Fang, R., Min Jou, W., Devos, R., Huylebroeck, D., Saman, E., and Fiers, W., Nature 286, 771, (1980).
9. Gerhard, W., Yewdell, J., and Frankel, M., in "Structure and Variation in Influenza Viruses" (W.G. Laver and G. Air, eds.), 273, Elsevier North Holland, (1980).
10. Webster, R.G., and Laver, W.G., Virology 104, 149, (1980).
11. Wilson, I.A., Skehel, J.J., and Wiley, D.C., Nature 289, 366, (1981).
12. Wiley, D.C., Wilson, J.A., and Skehel, J.J., Nature 289, 373, (1981).
13. World Health Organization. Bull. Wld. Hlth. Org. 58, 294, (1980).
14. Laver, W.G., and Webster, R.G., Virology 51, 383, (1973).
15. van Wyke, K.L., Hinshaw, V.S., Bean, W.J., and Webster, R.G., J. Virol., 35, 24, (1980).
16. Webster, R.G., and Berton, M.T., J. Gen. Virol. (In press, 1981).

THE HONG KONG HEMAGGLUTININ. STRUCTURAL RELATIONSHIPS BETWEEN THE HUMAN (H3) HEMAGGLUTININS AND THE HEMAGGLUTININ FROM THE PUTATIVE PROGENITOR STRAIN A/DUCK/UKRAINE/1/63 (HAV7)

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ABSTRACT

The structural relationship between the hemagglutinins from the human Hong Kong variants (H3) and the putative progenitor strain A/duck/Ukraine/1/63 (Hav7) has been investigated. Amino acid composition and sequence analysis showed that the Hav7 hemagglutinin closely resembled the H3 hemagglutinin in structure. It exhibited extensive sequence homology with the H3 hemagglutinins and contained the glycosylated 10 residue extension that is characterisitc of the N-terminal end of the Hong Kong HA1. Twenty-three differences were found between the hemagglutinin of A/duck/Ukraine/1/63 and the early Hong Kong variant A/Aichi/2/68; of these fifteen occurred in HA1 (at positions 4,25,62,81,92,135,137, 144,145,182,186,193,226,228 and 309), and eight occurred in HA2 (at positions 2,67,71,106,132,133,154 and 161). The oligosaccharide distribution was also different with the A/duck/Ukrainehemagglutinin containing only five carbohydrate side chains at positions 8,22,38,165 and 285 on HA1. It lacked the oligosaccharide units at positions 81 on HA1 and 154 on HA2.

The large number of amino acid sequence differences between the Hav7 and the early H3 hemagglutinins suggest that the Hong Kong hemagglutinin gene did not come directly from A/duck/Ukraine/1/63 but from a virus derived from it by antigenic drift during the period 1963 to 1968.

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I. INTRODUCTION

There has been considerable speculation about the mechanisms of antigenic variation in influenza virus (see refs. 1 & 2 for reviews), the number and nature of antigenic determinants on the influenza virus hemagglutinin (3-6) and the way these determinants change during antigenic shift and drift. Fazekas de St. Groth (1,7,8) suggested that the members of each sub-type could be arranged in hierarchic order and that antigenic drift involved the sequential replacement, at a single locus, of hydrophobic amino acids of increasing side chain surface area. The transition to new sub-types, antigenic shift, was believed to be an extension of the point mutation process occurring during antigenic drift, and to involve the production of a reservoir of bridging strains or double mutants in which substitutions at a second locus in the antigenic determinant had occurred.

The recent findings on the primary (9-16), tertiary (17, 18) and nucleotide sequences (19-23) of several natural or artificially selected antigenic variants from the Hong Kong sub-type have shown that this theory is incorrect. Antigenic drift does not involve the sequential replacement of amino acid residues at a single locus. In most cases, once a nucleotide or amino acid residue changed during antigenic drift it did not change again in subsequent mutant generations. Instead, as antigenic drift proceeded, amino acid substitutions accumulated at an increasing number of different loci (see ref. 24 for detailed review).

The Fazekas bridging strain hypothesis (1,7,8) for antigenic shift has also been challenged. The large differences in the amino acid and gene sequences of the hemagglutinins from different sub-types (11,19,25-27) compared to the small number of sequence changes seen within sub-types show that antigenic shift cannot involve an extension of the point mutation process operating during antigenic drift, but must occur by some other means.

Genetic re-assortment has been proposed as one possible mechanism for the production of new human pandemics (28) and considerable evidence supports the operation of genetic re-assortment in nature (1,29-32). Base sequence homology studies (33) showed that the Asian sub-type H2N2 was derived from the H1N1 sub-type by genetic re-assortment in which four of the H1N1 gene segments (bands 1,5,7 and 8) were retained while the other four (bands 2,3,4 and 6) were derived from an as yet unknown virus. The Hong Kong sub-type appeared to retain seven gene segments from the preceding H2N2 sub-type with only the hemagglutinin gene coming from some other

Table 1. Amino Acid Compositions of A/duck/Ukraine/1/63 Hemagglutinin Chains

Residue	HA1					HA2				
	duck/63 (Hav7)	X-31 (H3)	Jap/57 (H2)	Bel/42 (H0/H1)	FPV (Hav1)	duck/63	X-31 (H3)	Jap/57 (H2)	Bel/42 (H0/H1)	FPV (Hav1)
Aspartic acid	43.6 (46)	44	34	37.4	31	30.4	30	31	29.9	30
Threonine[a]	29.4 (30)	30	29	22.6	27	9.2	8	7	10.2	11
Serine[a]	25.9 (28)	30	23	27.6	30	10.3	9	15	17.1	10
Glutamic acid	22.9 (25)	23	33	34.4	32	29.7	29	25	27.3	30
Proline[b]	20.8 (19)	20	18	17.7	14	0.3	0	1	2.6	1
Glycine	24.6 (25)	26	28	24.6	33	19.9	19	22	21.3	17
Alanine	14.9 (14)	13	10	15.8	14	13.9	13	11	12.0	15
Valine[b]	19.2 (19)	20	18	17.7	17	9.6	8	13	12.8	10
Methionine	4.2 (4)	4	6	2.0	5	4.0	4	10	3.4	5
Isoleucine[b]	23.8 (25)	23	18	20.6	19	18.6	21	11	12.8	19
Leucine	22.0 (21)	23	29	25.6	21	15.6	19	18	19.6	17
Tyrosine	10.6 (11)	11	10	13.8	8	6.7	7	11	9.4	7
Phenylalanine	11.1 (10)	10	8	10.8	13	11.6	11	10	9.4	12
Histidine	6.5 (6)	6	10	7.9	7	5.0	5	4	3.4	4
Lysine	15.4 (14)	15	22	17.7	17	14.6	14	15	16.2	12
Arginine	18.5 (16)	15	12	13.8	19	10.7	11	8	5.1	9
Tryptophan[a]	6.6 (6)	6	7	ND	3	4.4	5	4	ND	5
Cysteine[c]	8.0 (9)	9	9	6.9	9	6.8	8	6	3.4	7
Total	328	328	324	317 + Trp	319	221	221	222	218 + Trp	221

a A/duck/Ukraine values corrected by extrapolation to zero time.
b A/duck/Ukraine values after 72 hr hydrolysis.
c A/duck/Ukraine values after 24 and 48 hour hydrolysis of performic acid oxidized samples.

The amino acid compositions for X-31 (11), Jap/57 (26) and FPV (25) are based on the final sequences. The data for duck/Ukraine/1/63 is from (36,37). Values in parenthesis indicate number of residues found in the sequence (37).

source. Laver and Webster (28) suggested that the 1968 Hong Kong hemagglutinin may have been donated by an animal virus related to A/equine/Miami/1/63 (Heq2,Neq2) or a bird virus related to A/duck/Ukraine/1/63 (Hav7,Neq2) since both viruses, isolated some five years before, showed antigenic (34) and peptide map similarities (28) to the Hong Kong hemagglutinin. Since the base sequence homology between Heq2 and H3 is only 80% (35) compared to the 92% homology between Hav7 and H3 (33) the duck/Ukraine/1/63 virus would appear to be the more likely donor of the Hong Kong hemagglutinin gene.

To investigate this possibility we have determined the primary structure of the hemagglutinin from A/duck/Ukraine/1/63 (36,37) and compared it with the structures of other influenza hemagglutinins.

II. METHODS

a. Virus strain and protein preparation. The virus strain used and the procedures employed in virus cultivation, purification, hemagglutinin isolation and separation into HA1 and HA2 have been previously described (9,37).

b. Peptide fragmentation. The procedures employed in reduction, carboxymethylation with 2-^{14}C-iodoacetic acid, cyanogen bromide cleavage and chromatographic separation of the resultant peptides; enzymic hydrolysis and peptide purification by gel chromatography and high voltage paper electrophoresis have been fully described (9,38).

c. Amino acid sequence determination. The strategy for sequence determination by the comparative peptide approach has been fully described (11). Automated sequence analyses, manual dansyl-Edman degradation and amino acid analyses were as previously described (9,11).

III. RESULTS

A. Amino Acid Composition

The amino acid compositions for A/duck/Ukraine/1/63 HA1 and HA2 are shown in Table 1 along with the available compositions for representatives of the three human sub-types H0/H1, H2 and H3 and fowl plague hemagglutinin Hav1 for comparison. The results show that the duck/Ukraine hemagglutinin closely resembles the H3 sub-type in amino acid composition. This is most easily seen by comparing the number of methionine and histidine residues in Hav7 and H3, and the aspartic acid/glutamic acid, glycine/alanine and tyrosine/phenylalanine ratios.

Table 2. Amino Acid Composition and Sequence of A/duck/Ukraine/1/63 Hemagglutinin Peptides That Differed from the Corresponding Peptides in A/Aichi/2/68

Peptide	N-Terminus	Mobility[c]	Composition[b]	Sequence[a]	Position[a]
HA1.CN1 T1.Th2	Leu	-0.18	Asx 2.9, Thr 1.2, Ser 1.8, Gly 1.1, Leu 1.0, GlcNAc 1.5	L S G N D $\overset{*}{N}$ S T	3-10
T1.Th6	Ile	+0.52	Val 0.7, Ile 0.7, Lys 1.0	I V K	25-27
T2.Sa1	Thr	-0.69	Asx 2.0, Thr 1.9, Glx 2.1, Ile 2.0	T I T D D Q I E	28-35
T4	Ile	0	Asx 0.9, Gly 1.0, Ile 0.7, Leu 0.7, Arg 1.1	I L D G R	58-62
T5.Th4	Leu	-0.54	CMCys 0.9, Asx 2.0, Pro 0.9, Gly 1.1, Val 0.9, Leu 1.0, His 1.1	L G D P H C D V	71-78
T5.Th5	Phe	-0.58	Asx 2.1, Thr 0.9, Glx 2.0, Phe 0.9, Trp	F Q D E T W D	79-85
T6 C1	Ser	0	Asx 1.1, Ser 0.9, Ala 1.1, Phe 0.9	S N A F	91-94
T7.Th5	Val	-0.23	CMCys 0.9, Asx 1.1, Thr 1.0, Ser 1.9, Glx 2.1, Gly 1.0, Ala 1.0, Val 1.0, Lys 1.0	V T Q N G E S S A C K	130-140
T8	Gly	0	Asx 1.1, Ser 0.8, Pro 1.1, Gly 1.9, Ala 0.9, Phe 2.0, Arg 1.1	G P A D G F F S R	142-150
T10	Ser	0	Asx 1.0, Thr 1.8, Ser 1.8, Pro 1.1, Gly 1.0, Val 2.0, Leu 1.0, Tyr 1.0, HSer 0.9, GlcNAc 0.9	S G S T Y P V L $\overset{*}{N}$ V T M	157-168
CN2.T2.P2.Th3	Ile	-0.23	Asx 1.9, Thr 1.9, Glx 3.1, Ile 1.1, Leu 0.6	I T N Q E Q T N L	186-194
T7	Gly	+0.50	Ser 0.9, Glx 0.9, Gly 2.1, Arg 1.1	G Q S G R	225-229
CN3.T3	Tyr	+0.63	Ile 1.1, Tyr 1.0, Lys 0.9	Y I K	308-310
HA2.CN4.	Gly	-	Asx 1.1, Glx 2.0, Gly 4.6, Ala 2.0, Ile 2.9, Phe 2.0, Trp 1.2, HSer 0.9	G I F G A I A G F I E N G W E G M	1-17
CN2.T7	Phe	+0.32	Glx 2.0, Ile 0.9, Phe 0.8, His 0.8, Lys 1.0	F H Q I Q K	63-68
T8	Glu	-0.48	Glx 3.0, Gly 1.1, Val 1.0, Phe 2.0, Arg 1.0	E F F E V E G R	69-76
T11.Sa3	Asn	+0.50	Asx 1.0, Glx 1.0, Lys 0.8	N Q K	104-106
CN2/3.T12	Thr	-0.70	CMCys 0.6, Asx 3.0, Glx 2.0, Gly 2.3, Ala 1.0, Ile 0.9, Phe 0.9, Lys 1.1	E N A E D I G N G C F K	128-139
CN1.T4/5	Lys	0.05	Asx 1.8, Thr 0.8, Gly 1.0, Ile 1.0, Tyr 1.7, His 0.6, Lys 0.9, Arg 0.9	K G T Y D H D I Y R	154-163

a. For the location of these peptides and their comparison with A/Aichi/2/68 see Fig. 2.
b. Abbreviations used: CMCys, carboxymethylcysteine; GlcNAc, N-acetylglucosamine; HSer, homoserine.
c. Electrophoretic mobility at pH 6.5 relative to Aspartic acid (m = -1.0).

B. Amino Acid Sequence

The new classification of influenza viruses recognises twelve sub-types of hemagglutinin based on the absence of any common antigenic determinants in double diffusion tests (see 39). The amino acid sequences for the N-terminal region of the HA1 polypeptide chain from the twelve sub-types (27) are shown in Fig. 1 and directly compared with the sequence data obtained from A/duck/Ukraine/1/63 (36,37). The results clearly show that A/duck/Ukraine (Hav7) belongs to the Hong Kong H3 sub-type. It contains an N-terminal blocking group on HA1 (42) which is presumed to be a cyclised glutamine residue (pyrrolidone carboxylic acid) as found in the H3 hemagglutinin (9,11) since the blocking group is effectively removed by pyroglutamic acid aminopeptidase (36). It also contains the glycosylated 10 residue extension characteristic of the H3 hemagglutinins. All other sub-type hemagglutinins start at residue 11 except Hav4 which is believed to start at position 5 (Fig. 1).

The N-terminal sequence data for the HA2 polypeptide further shows that the Hav7 structure is very similar to that of the H3 viruses (Fig. 1). The HA2 chains from the other sub-type variants examined to date differ from the Hav7/H3 sequences at residues 12,15,17,18,24,25,27,29,30,32,34,38 and 39, depending on strain.

These N-terminal sequences demonstrated that the Hav7 and H3 hemagglutinins were closely related (36) and suggested that the complete sequence of the Hav7 hemagglutinin could be rapidly determined by the comparative peptide approach used to determine the structure of the early Hong Kong variant A/Aichi/2/68 (11). This involved cyanogen bromide cleavage of HA1 and HA2 followed by tryptic digestion of the isolated cyanogen bromide peptides. Large tryptic peptides were further digested with thermolysin, chymotrypsin, pepsin or S. aureus protease. The amino acid sequence of each resulting peptide was deduced by comparing its chromatographic behaviour, electrophoretic mobility, amino acid composition and N-terminus with that of the corresponding peptides from the hemagglutinins of Aichi/2/68 and Mem/102/72 whose structures are known (9-11). Those peptides which contained differences were sequenced directly and are listed in Table 2.

The amino acid sequence of A/duck/Ukraine/1/63 hemagglutinin is shown in Fig. 2. The sequence is complete except for the highly aggregated hydrophobic region (residues 180-207) near the C-terminal end of HA2, that could not be resolved by peptide sequencing. The Hav7 hemagglutinin is very similar to those of the human Hong Kong strains (9-11,19-23). It contains 328 amino acid residues in HA1 including four

HA1

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H1 (HO,H1) A/PR/8/34                                                    Asp Thr Ile Cys Ile Gly Tyr His Ala Asn
H2 (H2) A/R15~/57                                                       Asp Gln Ile Cys Ile Gly Tyr His Ala Asn
H5 (Hav5) A/Shearwater/Aust/75                                          Asp Gln Ile Cys Ile Gly Tyr His Ala Asn
H11 (Hav3) A/duck/Mem/546/76                                            Asp Glu Ile Cys Ile Gly Tyr Leu Ser Asn
H6 (Hav6) A/Shearwater/Aust/72                                          Asp Lys Ile Cys Ile Gly Tyr His Ala Asn
H8 (Hav8) A/turkey/Ont/6118/68                                          Asp Arg Ile Cys Ile Gly Tyr Gln Ser Asn
H9 (Hav9) A/turkey/Wis/1/66                                             Asp Lys Ile Cys Ile Gly Tyr Gln Ser Thr
H12 (Hav10) A/duck/Alb/60/76                                            Asp Lys Ile Cys Ile Gly Tyr Gln Thr Asn
H7 (Hav1) A/turkey/Oregon/71                                            Asp Lys Ile Cys Leu Gly His His Ala Val
H10 (Hav2) A/duck/Manitoba/53                                           Asp Lys Ile Cys Leu Gly His His Ala Val
H4 (Hav4) A/duck/Alb/28/76                        Gln Asn Tyr Thr Gly Asn Pro Val Ile Cys Leu Gly His His Ala Val
H3 (H3) A/Mem/1/71          Gln Tyr Leu Pro Gly Asn Asp Asn Ser Thr Ala Thr Leu Cys Leu Gly His His Ala Val

Hav7 A/duck/Ukraine/1/63    Gln Asp Leu Ser Gly Asn Asp Asn Ser Thr Ala Thr Leu Cys Leu Gly His His Ala Val
                                        5                  10                  15                  20


H1 (HO,H1) A/PR/8/34        Asn Ser Thr Asp Thr Val Asp Thr Val Leu Glu Lys Asn Val Thr Val Thr His Ser Val
H2 (H2) A/R15~/57           Asn Ser Thr Glu Lys Val Asp Thr Ile Leu Glu Arg Asn Val Thr Val Thr His Ala Lys
H5 (Hav5) A/Shearwater/Aust/75 Asn Ser Thr Glu Gln Val Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln
H11 (Hav3) A/duck/Mem/546/76 Asn Ser Thr Glu Lys Val Asp Thr Ile Ile Glu Ser Asn Val Thr Val Thr Ser Ser Val
H6 (Hav6) A/Shearwater/Aust/72 Asn Ser Thr Thr Gln Ile Asp Thr Ile Leu Glu Lys Asn Val Thr Val Thr His Ser Val
H8 (Hav8) A/turkey/Ont/6118/68 Asn Ser Thr Asp Thr Val Asn Thr Leu Thr Glu Gln Asn Val Pro Val Thr Gln Thr Met
H9 (Hav9) A/turkey/Wis/1/66 Asn Ser Thr Glu Thr Val Asp Thr Leu Thr Glu Ser Asn Val Pro Val Thr His Thr Lys
H12 (Hav10) A/duck/Alb/60/76 Asn Ser Thr Glu Thr Val Asn Thr Leu Ser Glu Leu Asn Val Pro Val Thr Gln Val Glu
H7 (Hav1) A/turkey/Oregon/71 Ala Asn Gly Thr Lys Val Asn Thr Leu Thr Glu Arg Gly Ile Glu Val Val Asn Ala Thr
H10 (Hav2) A/duck/Manitoba/53 Pro Asn Gly Ile Ile Val Lys Thr Leu Thr Asn Glu Lys Glu Glu Val Thr Asn Ala Thr
H4 (Hav4) A/duck/Alb/28/76 Ser Asn Gly Thr Met Val Lys Thr Leu Thr Asp Asp Gln Val Glu Val Val Thr Ala Gln
H3 (H3) A/Mem/1/71          Pro Asn Gly Thr Leu Val Lys Thr Ile Thr Asn Asp Gln Ile Glu Val Thr Asn Ala Thr

Hav7 A/duck/Ukraine/1/63    Pro Asn Gly Thr Ile Val Lys Thr Ile Thr Asp Asp Gln Ile Glu Val Thr Asn Ala Thr
                                        25                  30                  35                  40
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HA2

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H1 (HO,H1) A/Bel/42                Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu Gly Gly Trp Thr Gly Met
H2 (H2) A/Jap/305/57               Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu Gly Gly Trp Glu Gly Met Val Asp Gly
H5
H11
H6 (Hav6) A/Shearwater/E.Aust/72   Gly
H8
H9
H12
H7 (Hav1) A/FPV/Rostock            Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu Asn Gly Trp Glu Gly Leu Val Asp Gly
H10 (Hav2) A/chick/Germany/49      Gly Leu Phe Gly Ala Ile
H4
H3 (H3) A/Aichi/2/68               Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu Asn Gly Trp Glu Gly Met Ile Asp Gly

Hav7 A/duck/Ukraine/1/63           Gly Ile Phe Gly Ala Ile Ala Gly Phe Ile Glu Asn Gly Trp Glu Gly Met Ile Asp Gly
                                           5                  10                  15                  20


H1
H2 (H2) A/Jap/305/57               Trp Tyr Gly Tyr His His Ser Asn Asp Gln Gly Ser Gly Tyr Ala Ala Asp Lys Glu Ser
H5
H11
H6
H8
H9
H12
H7 (Hav1) A/FPV/Rostock            Trp Tyr Gly Phe Arg His Gln Asn Ala Gln Gly Glu Gly Thr Ala Ala Asp Tyr Lys Ser
H10
H4
H3 (H3) A/Aichi/2/68               Trp Tyr Gly Phe Arg His Gln Asn Ser Glu Gly Thr Gly Gln Ala Ala Asp Leu Lys Ser

Hav7 A/duck/Ukraine/1/63           Trp Tyr Gly Phe Arg His Gln Asn Ser Glu Gly Thr Gly Gln Ala Ala Asp Leu Lys Ser
                                           25                  30                  35                  40
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FIGURE 1. Comparison of N-terminal sequences of HA1 and HA2 from A/duck/Ukraine/1/63 (Hav7) with the available data for representative strains from the twelve known hemagglutinin sub-types (27). The start of HA1 in sub-types H5,H11,H8,H9, H10 and H12 is inferred by homology with H1,H2,H6 and H7. (11,41,42,56). The start of H4 is inferred by homology with H3. The comparative H1-H12 sequences are those from the cDNA sequences obtained by Air et al. (27). The HA2 data is from refs. 11,25,26,42,57 and 58. The glycosylation sites are underlined.

methionines (residues 168,260,268 and 320) and nine half-cystines (residues 14,52,64,76,97,139,277,281 and 305). A/duck/Ukraine HA2 contained four methionine residues at positions 17,115,133 and presumably 207 in the aggregated hydrophobic tail region, although a partial Ile for Met replacement was observed at residue 133. Six of the eight half-cystine residues in HA2 were found at positions 137,144, 148,210,217 and 220, the other two being located in the aggregated hydrophobic tail region (residues 180-207).

When compared with the structure of the hemagglutinin from the early Hong Kong variant A/Aichi/2/68 derived by DNA cloning and sequencing (21) twenty-three differences can be detected. Of these, fifteen occur in HA1 (positions 4,25,62, 81,92,135,137,144,145,182,186,193,226,228 and 309) and eight occur in HA2 (positions 2,67,71,106,132,133,154 and 161). The Ile for Met replacement at 133 in HA2 was only partial. When compared with the amino acid sequence obtained for Aichi/2/68 by peptide sequencing (11) four additional differences can be seen at positions 2,31,78 and 158 in HA1 but not the differences at 182 in HA1 or 132 in HA2 (see Fig. 4). The Aichi/2/68 sequence obtained by peptide analyses (11), like all other Hong Kong strains studied to date (9,10,19,20, 22) had Val not Ile at position 182 in HA1 and Gly not Glu at position 132 in HA2 (see Fig. 4).

From an analysis of the three-dimensional structure of the Hong Kong hemagglutinin and their location of the amino acid substitutions that have occurred during antigenic drift in natural and laboratory selected variants. Wiley et al. (18) suggested that there were four antigenic regions, A,B,C and D on the Hong Kong hemagglutinin and that each major epidemic strain contained at least one amino acid substitution in each of these four regions. It is interesting to note that of the 23 to 25 differences in amino acid sequence found between the A/duck/Ukraine/1/63 hemagglutinin and Aichi/2/68 (11,21) four occur in antigenic region A (residues 135,137, 144 and 145) and three occur in region B (residues 158,186 and 193). There are no substitutions at residues that appear to be involved in antigenic regions C and D.

FIGURE 2. Amino acid sequences of A/duck/Ukraine/1/63 (37) and A/Aichi/2/68 (11,21) hemagglutinins. Methionine residues occur at positions 168,260,268 and 320 in HA1 and 17,115,133 and the insoluble hydrophobic tail region of HA2. A partial replacement of Ile for Met at position 133 in A/duck/Ukraine/ 1/63 HA2 was observed. The residues that differ are enclosed in boxes.

```
                     HA1                         *
A/duck/Ukraine/1/63 Glx Asp Leu Ser Gly Asn Asp Asn Ser Thr Ala Thr Leu Cys Leu Gly His His Ala Val
Aichi/2/68 (X-31)   GLX ASP LEU PRO GLY ASN ASP ASN SER THR ALA THR LEU CYS LEU GLY HIS HIS ALA VAL
                                                 *       10                                      20

     *
Pro Asn Gly Thr Ile Val Lys Thr Ile Thr Asp Asp Gln Ile Glu Val Thr Asn Ala Thr Glu Leu Val Gln Ser
PRO ASN GLY THR LEU VAL LYS THR ILE THR ASP ASP GLN ILE GLU VAL THR ASN ALA THR GLU LEU VAL GLN SER
     *                                  30                                      40

Ser Ser Thr Gly Lys Ile Cys Asn Asn Pro His Arg Ile Leu Asp Gly Arg Asp Cys Thr Leu Ile Asp Ala Leu
SER SER THR GLY LYS ILE CYS ASN ASN PRO HIS ARG ILE LEU ASP GLY ILE ASP CYS THR LEU ILE ASP ALA LEU
                50                                      60                                      70

Leu Gly Asp Pro His Cys Asp Val Phe Gln Asp Glu Thr Trp Asp Leu Phe Val Glu Arg Ser Asn Ala Phe Ser
LEU GLY ASP PRO HIS CYS ASP VAL PHE GLN ASN GLU THR TRP ASP LEU PHE VAL GLU ARG SER LYS ALA PHE SER
                                        80  *                                   90

Asn Cys Tyr Pro Tyr Asp Val Pro Asp Tyr Ala Ser Leu Arg Ser Leu Val Ala Ser Ser Gly Thr Leu Glu Phe
ASN CYS TYR PRO TYR ASP VAL PRO ASP TYR ALA SER LEU ARG SER LEU VAL ALA SER SER GLY THR LEU GLU PHE
                100                                     110                                     120

Ile Thr Glu Gly Phe Thr Trp Thr Gly Val Thr Gln Asn Gly Glu Ser Ser Ala Cys Lys Arg Gly Pro Ala Asp
ILE THR GLU GLY PHE THR TRP THR GLY VAL THR GLN ASN GLY GLY SER ASN ALA CYS LYS ARG GLY PRO GLY SER
                                        130                                     140
                                                                                 *
Gly Phe Phe Ser Arg Leu Asn Trp Leu Thr Lys Ser Gly Ser Thr Tyr Pro Val Leu Asn Val Thr Met Pro Asn
GLY PHE PHE SER ARG LEU ASN TRP LEU THR LYS SER GLY SER THR TYR PRO VAL LEU ASN VAL THR MET PRO ASN
                150                                     160                      *              170

Asn Asp Asn Phe Asp Lys Leu Tyr Ile Trp Gly Val His His Pro Ile Thr Asn Gln Glu Gln Thr Asn Leu Tyr
ASN ASP ASN PHE ASP LYS LEU TYR ILE TRP GLY ILE HIS HIS PRO SER THR ASN GLN GLU GLN THR SER LEU TYR
                                        180                                     190

Val Gln Ala Ser Gly Arg Val Thr Val Ser Thr Arg Arg Ser Gln Gln Thr Ile Ile Pro Asn Ile Gly Ser Arg
VAL GLN ALA SER GLY ARG VAL THR VAL SER THR ARG ARG SER GLN GLN THR ILE ILE PRO ASN ILE GLY SER ARG
                200                                     210                                     220

Pro Trp Val Arg Gly Gln Ser Gly Arg Ile Ser Ile Tyr Trp Thr Ile Val Lys Pro Gly Asp Val Leu Val Ile
PRO TRP VAL ARG GLY LEU SER SER ARG ILE SER ILE TYR TRP THR ILE VAL LYS PRO GLY ASP VAL LEU VAL ILE
                                        230                                     240

Asn Ser Asn Gly Asn Leu Ile Ala Pro Arg Gly Tyr Phe Lys Met Arg Thr Gly Lys Ser Ser Ile Met Arg Ser
ASN SER ASN GLY ASN LEU ILE ALA PRO ARG GLY TYR PHE LYS MET ARG THR GLY LYS SER SER ILE MET ARG SER
                250                                     260                                     270
                                                     *
Asp Ala Pro Ile Asp Thr Cys Ile Ser Glu Cys Ile Thr Pro Asn Gly Ser Ile Pro Asn Asp Lys Pro Phe Gln
ASP ALA PRO ILE ASP THR CYS ILE SER GLU CYS ILE THR PRO ASN GLY SER ILE PRO ASN ASP LYS PRO PHE GLN
                                        280              *                      290

Asn Val Asn Lys Ile Thr Tyr Gly Ala Cys Pro Lys Tyr Ile Lys Gln Asn Thr Leu Lys Leu Ala Thr Gly Met
ASN VAL ASN LYS ILE THR TYR GLY ALA CYS PRO LYS TYR VAL LYS GLN ASN THR LEU LYS LEU ALA THR GLY MET
                300                                     310                                     320
                                        HA2
Arg Asn Val Pro Glu Lys Gln Thr         Gly Ile Phe Gly Ala Ile Ala Gly Phe Ile Glu Asn Gly Trp Glu
ARG ASN VAL PRO GLU LYS GLN THR         GLY LEU PHE GLY ALA ILE ALA GLY PHE ILE GLU ASN GLY TRP GLU
                                                                        10

Gly Met Ile Asp Gly Trp Tyr Gly Phe Arg His Gln Asn Ser Glu Gly Thr Gly Gln Ala Ala Asp Leu Lys Ser
GLY MET ILE ASP GLY TRP TYR GLY PHE ARG HIS GLN ASN SER GLU GLY THR GLY GLN ALA ALA ASP LEU LYS SER
                20                                      30                                      40

Thr Gln Ala Ala Ile Asp Gln Ile Asn Gly Lys Leu Asn Arg Val Ile Glu Lys Thr Asn Glu Lys Phe His Gln
THR GLN ALA ALA ILE ASP GLN ILE ASN GLY LYS LEU ASN ARG VAL ILE GLU LYS THR ASN GLU LYS PHE HIS GLN
                                        50                                      60

Ile Gln Lys Glu Phe Phe Glu Val Glu Gly Arg Ile Gln Asp Leu Glu Lys Tyr Val Glu Asp Thr Lys Ile Asp
ILE GLU LYS GLU PHE SER GLU VAL GLU GLY ARG ILE GLN ASP LEU GLU LYS TYR VAL GLU ASP THR LYS ILE ASP
                70                                      80                                      90

Leu Trp Ser Tyr Asn Ala Glu Leu Leu Val Ala Leu Glu Asn Gln Lys Thr Ile Asp Leu Thr Asp Ser Glu Met
LEU TRP SER TYR ASN ALA GLU LEU LEU VAL ALA LEU GLU ASN GLN HIS THR ILE ASP LEU THR ASP SER GLU MET
                                        100                                     110

Asn Lys Leu Phe Glu Lys Thr Arg Arg Gln Leu Arg Glu Asn Ala Glu Asp Ile Gly Asn Gly Cys Phe Lys Ile
ASN LYS LEU PHE GLU LYS THR ARG ARG GLN LEU ARG GLU ASN ALA GLU GLU MET GLY ASN GLY CYS PHE LYS ILE
                120                                     130                                     140

Tyr His Lys Cys Asp Asn Ala Cys Ile Glu Ser Ile Arg Lys Gly Thr Tyr Asp His Asp Ile Tyr Arg Asp Glu
TYR HIS LYS CYS ASP ASN ALA CYS ILE GLU SER ILE ARG ASN GLY THR TYR ASP HIS ASP VAL TYR ARG ASP GLU
                                        150         *                           160

Ala Leu Asn Asn Arg Phe Gln Ile Lys Gly Val Glu Leu Lys ----------------------------------------
ALA LEU ASN ASN ARG PHE GLN ILE LYS GLY VAL GLU LEU LYS SER GLY TYR LYS ASP TRP ILE LEU TRP ILE SER
                170                                     180                                     190

------- insoluble ------------------------------------------------ Trp Ala Cys Gln Arg Gly Asn Ile
PHE ALA ILE SER CYS PHE LEU LEU CYS VAL VAL LEU LEU GLY PHE ILE MET TRP ALA CYS GLN ARG GLY ASN ILE
                                        200                                     210

Arg Cys Asn Ile Cys Ile
ARG CYS ASN ILE CYS ILE
                220
```

A. Oligosaccharide Distribution and Composition

A/duck/Ukraine/1/63 hemagglutinin contains only five oligosaccharide units and these are attached at asparagine residues 8,22,38,165 and 285. It lacks the glycosylation sites at position 81 in HA1 and 154 in HA2 owing to Asn to Asp and Asn to Lys replacements respectively (see. Fig. 4). The late Hong Kong variants A/Vic/3/75 (22), A/Texas/1/77 (Moss unpublished) and A/Bangkok/79 (23) also lack the glycosylation site at Asn_{81} owing to a Thr to Lys substitution at position 83. These late Hong Kong strains however have a new potential glycosylation site at Asn_{63} which occupies a very similar position to Asn_{81} on the three-dimensional structure (17). A/duck/Ukraine does not have this new glycosylation site and thus lacks carbohydrate on this region of the molecule. In this respect it resembles the hemagglutinins from the other eleven sub-types none of which possess glycosylation sites at 63 or 81 (27). A/duck/Ukraine is the only hemagglutinin studied to date that lacks the glycosylation site at residue 154 on HA2.

The sugar compositions for the five oligosaccharide units on A/duck/Ukraine/1/63 hemagglutinin are shown in Table 3 and compared with the corresponding oligosaccharide units on Aichi/2/68 (11) and Mem/102/72 (43). The results show that in addition to the differences seen in the distribution of glycosylation sites on the Hav7 and H3 hemagglutinins, there are also differences in the sugar compositions of the oligosaccharide units attached at homologous positions on the different hemagglutinins. In all three strains the carbohydrate units at residues 8 and 22 are of the N-acetyllactosamine ("complex") type while those at 285 are of the oligomannoside ("simple") type. The sugar units at residues 38 in duck/Ukraine/63 and Aichi/2/68 and residue 165 in duck/Ukraine/63 also resemble the oligomannoside type but contain small, but significant, amounts of galactose and fucose. As pointed out previously (11), this may reflect partial processing of the mannose-rich precursor sugar moiety (see 44) during hemagglutinin biosynthesis. In both the human Hong Kong influenza strains, Aichi/68 and Mem/72, the sugar units at position 165 of HA1 were "simple" containing two residues of N-acetylglucosamine and six to nine residues of mannose.

Examination of the amino acid sequences of duck/Ukraine/63, Aichi/2/68 and Mem/102/72 (Fig. 4) shows that all potential glycosylation sites, Asn-X-$\begin{matrix}\text{Ser}\\\text{Thr}\end{matrix}$ sequences, do have carbohydrate attached. This is in contrast to the situation with the A/Jap/305/57 Asian hemagglutinin which has carbohydrate attached at only five of the nine potential glycosylation sites on the protein (45,46). Of the four sites not glyco-

Table 3. Carbohydrate Composition of the Oligosaccharide Units on A/duck/Ukraine/1/63 and Two Hong Kong (H3) Hemagglutinins

		Sugar composition (moles/mole of peptide)											
		A/duck/Ukraine/1/63				Aichi/2/68				Mem/102/72			
Polypeptide	Asparagine Residue	GlcNAc	Man	Gal	Fuc	GlcNAc	Man	Gal	Fuc	GlcNAc	Man	Gal	Fuc
HA1	8	2.9	1.9	0.7	0.4	4.3	5.3	2.6	1.7	4.1	4.1	4.8	1.8
	22	2.0	1.7	2.1	1.7	4.3	3.3	2.3	1.9	3.7	2.1	2.2	1.3
	38	1.8	3.7	0.3	0.1	2.1	6.2	0.6	0.3	4.0	5.4	2.5	0.6
	81	absent				3.8	3.4	3.1	0.9	2.9	2.5	2.1	0.2
	165	1.8	4.3	0.4	0.2	1.9	8.6	-	-	1.9	5.8	-	-
	285	1.8	5.9	-	-	1.9	4.4	-	-	2.0	5.1	-	-
HA2	154	absent				3.9 3.2	3.8 2.9	2.3 1.5	1.5 0.9	4.0	2.6	2.2	1.1

The data for duck/Ukraine is from ref. 37; for Aichi/2/68 from ref. 11 and for Mem/72 from ref. 43.

sylated in A/Jap/305/57, two occur adjacent to glycosylated residues, one contains an Asn Pro Ser sequence and one is located at the C-terminal end of HA2 probably inside the viral membrane (see 24). All carbohydrate units on A/Jap/305/57 HA1 and HA2 are of the N-acetyllactosamine ("complex") type (46).

IV. DISCUSSION

While the amino acid composition and sequence data clearly show that the Hav7 hemagglutinin belongs to the H3 sub-type (see also 39) it appears that the hemagglutinin gene of the 1968 Hong Kong sub-type was not directly donated by the A/duck/Ukraine/1/63 virus since their amino acid sequences differ at 15 to 18 positions in HA1 and 7 to 8 positions in HA2. The data suggest however that this hemagglutinin may have come from a virus derived from A/duck/Ukraine/1/63 by antigenic drift during the period 1963-1968. As shown in Fig. 3) the rate of drift required, in the period 1963-1968, to accommodate this number of changes between A/duck/Ukraine/1/63 and A/Aichi/2/68 is similar to the rate of drift found to occur from 1968 to 1979 in the human Hong Kong sub-type. Secondly, an examination of the sequence data available for other Hong Kong variants (Fig. 4) shows that the duck/Ukraine/1/63 hemagglutinin is identical to Aichi/2/68 at most of the positions that subsequently changed during antigenic drift in the human Hong Kong sub-type. This would be expected since antigenic drift involves the gradual accumulation of changes at different loci rather than sequential changes at a single or limited number of positions (see Fig. 4).

While there are no detailed studies on the precise rate and nature of amino acid sequence changes associated with antigenic drift in avian influenza viruses it has been shown by hemagglutinin inhibition, comparative RNA electrophoretic mobilities and RNA:RNA hybridization analyses that the hemagglutinin in several Hav7 viruses accumulated a large number of mutations during the period 1963 to 1976 (47). Furthermore the mutation rate of the so-called "antigenically stable" Sendai and vesticular stomatitis viruses (48) and of the "non-selected" non-structural and internal matrix proteins (27) of influenza virus are similar to that found in the influenza virus hemagglutinin. Thus there seems no reason to doubt that the rate of antigenic drift in duck influenza infections, (which do not appear to cause signs of disease (49)), is not at least as extensive as that found in human Hong Kong influenza. Thus it is suggested that a 1968 bird virus derived from A/duck/Ukraine/1/63 by antigenic drift, rather than A/duck/Ukraine/1/63 itself, was the virus

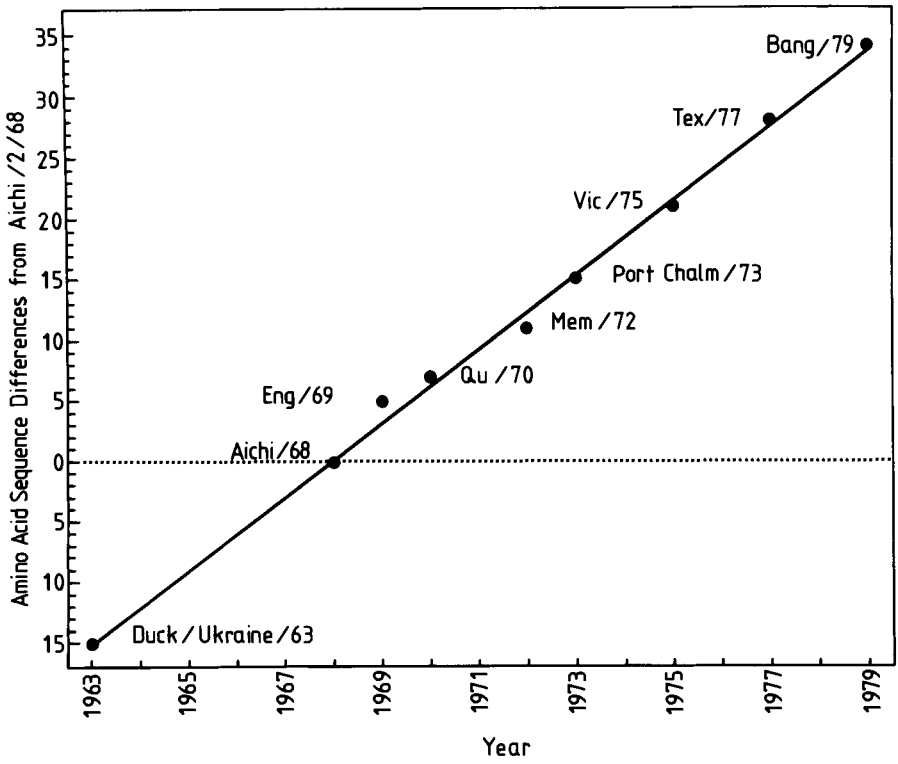
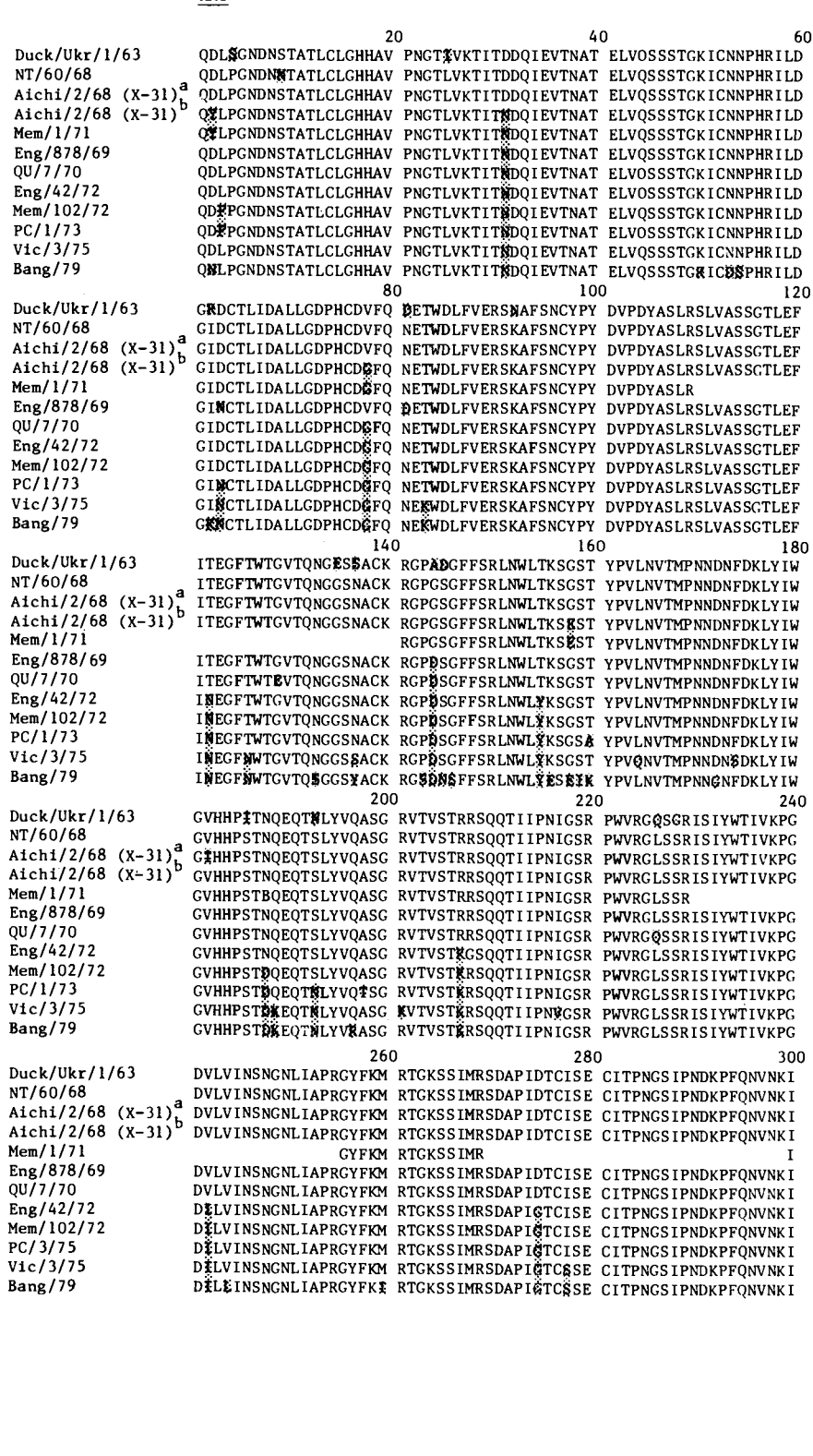


FIGURE 3. Frequency of amino acid substitutions in HA1. The number of differences in the amino acid sequences of the HA1 polypeptides of the various strains are shown. Duck/Ukraine/1/63 falls on the same line that shows the rate of change in the human Hong Kong sub-type, when extrapolated back to 1963. The data sources are duck/Ukraine/1/63 (37); Aichi/2/68 (11, 21); Eng/878/69, Qu/7/70 (20); Mem/102/72 (9,10); Port/Chalm/1/73 (23); Vic/3/75 (22); Texas/1/77 (Moss unpublished; see 24); Bang/79 (23).

that donated the human Hong Kong hemagglutinin. However it must be remembered that attempts to establish the origin of the hemagglutinins from human pandemics are hampered by the results of retrospective serology which showed that viruses containing Asian (H2) and Hong Kong (H3) hemagglutinins were present in the human population at the end of the last century (50,51).

While genetic re-assortment is one possible mechanism by which new human pandemics can be generated, other mechanisms as yet uncharacterized also exist, since the Russian influenza which re-emerged in 1977 was genetically indistinguishable from other HI strains present in the human population


HA1
20
40
60
Duck/Ukr/1/63
NT/60/68
Aichi/2/68 (X-31)a
Aichi/2/68 (X-31)b
Mem/1/71
Eng/878/69
QU/7/70
Eng/42/72
Mem/102/72
PC/1/73
Vic/3/75
Bang/79
80
100
120
140
160
180
200
220
240
260
280
300

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                                  320
Duck/Ukr/1/67          TYGACPKY‡KQNTLKLATGM RNVPEKQT
NT/60/68               TYGACPKYVKQNTLKLATGM RNVPEKQT
Aichi/2/68 (X-31)a     TYGACPKYVKQNTLKLATGM RNVPEKQT
Aichi/2/68 (X-31)b     TYGACPKYVKQNTLKLATGM RNVPEKQT
Mem/1/71               TYGACPKYVKQNTLKLATGM RNVPEKQT
Eng/878/69             TYGACPKYVKQNTLKLATGM RNVPEKQT
QU/7/70                TYGACPKYVKQNTLKLATGM RNVPEKQT
Eng/42/72              TYGACPKYVKQNTLKLATGM RNVPEKQT
Mem/102/72             TYGACPKYVKQNTLKLATGM RNVPEKQT
PC/3/75                TYGACPKYVKQNTLKLATGM RNVPEKQT
Vic/3/75               TYGACPKYVKQNTLKLATGM RNVPEKQT
Bang/79                TYGACPKYVKQNTLKLATGM RNVPEKQT
                       HA2
                                  20                  40                  60
Duck/Ukr/1/63          G‡FGAIAGFIENGWEGMIDG WYGFRHQNSEGTGQAADLKS TQAAIDQINGKLNRVIEKTN
NT/60/68               GLFGAIAGFIENGWEGMIDG WYGFRHQNSEGTGQAADLKS TQAAIDQINGKLNRVIEKTN
Aichi/2/68 (X-31)a     GLFGAIAGFIENGWEGMIDG WYGFRHQNSEGTGQAADLKS TQAAIDQINGKLNRVIEKTN
Aichi/2/68 (X-31)b     GLFGAIAGFIENGWEGMIDG WYGFRHQNSEGTGQAADLKS TQAAIDQINGKLNRVIEKTN
Mem/1/71                                    HQNSEGTGQAADLKS TQAAIDQINGKLNRVIEKTN
Mem/102/72             GLFGAIAGFIENGWEGMIDG WYGFRHQNSEGTGQAADLKS TQAAIDQINGKLNRVIEKTN
Vic/3/75               G‡FGAIAGFIENGWEGMIDG WYGFRHQNSEGTGQAADLKS TQAAIDQINGKLNRVIEKTN
Bang/79                G‡FGAIAGFIENGWEGMIDG WYGFRHQNSEGTGQAADLKS TQAAIDQINGKLNRVIEKTN
                                  80                  100                 120
Duck/Ukr/1/63          EKFHQIQKEF‡EVEGRIQDL EKYVEDTKIDLWSYNAELLV ALENQ‡TIDLTDSEMNKLFE
NT/60/68               EKFHQIEKEFSEVEGRIQDL EKYVEDTKIDLWSYNAELLV ALENQHTIDLTDSEMNKLFE
Aichi/2/68 (X-31)a     EKFHQIEKEFSEVEGRIQDL EKYVEDTKIDLWSYNAELLV ALENQHTIDLTDSEMNKLFE
Aichi/2/68 (X-31)b     EKFHQIEKEFSEVEGRIQDL EKYVEDTKIDLWSYNAELLV ALENQHTIDLTDSEMNKLFE
Mem/1/71               EKFHQIEKEFSEVEGRIQDL EKYVEDTK                           LFE
Mem/102/72             EKFHQIEKEFSEVEGRIQDL EKYVEDTKIDLWSYNAELLV ALENQHTIDLTDSEMNKLFE
Vic/3/75               EKFHQIEKEFSEVEGRIQDL EKYVEDTKIDLWSYNAELLV ALENQHTIDLTDSEMNKLFE
Bang/79                EKFHQIEKEFSEVEGRIQDL EKYVEDTKIDLWSYNAELLV ALENQHTIDLTDSEMNKLFE
                                  140                 160                 180
Duck/Ukr/1/63          KTRRQLRENAED‡GNGCFKI YHKCDNACIESIR‡GTYDHD ‡YRDEALNNRFQIKGVELK
NT/60/68               KTRRQLRENAEDMGNGCFKI YHKCDNACIESIRNGTYDHD VYRDEALNNRFQIKGVELKS
Aichi/2/68 (X-31)a     KTRRQLRENAE‡MGNGCFKI YHKCDNACIESIRNGTYDHD VYRDEALNNRFQIKGVELKS
Aichi/2/68 (X-31)b     KTRRQLRENAEDMGNGCFKI YHKCDNACIESIRNGTYDHD VYRDEALNNRFQIKGVELKS
Mem/1/71               KTRRQLRENAEDMGNGCFKI YHKCDNACIGSIRNGTYDHD VYRDEALNNRFQIKGVELK
Mem/102/72             KTRRQLRENAEDMGNGCFKI YHKCDNACIGSIRNGTYDHD VYRDEALNNRFQIKGVELKS
Vic/3/75               KTRRQLRENAEDMGNGCFKI YHKCDNACIGSIRNGTYDHD VYRDEALNNRFQIKGVELKS
Bang/79                KTRRQLRENAEDMGNGCFKI YHKCDNACIGSIRNGTYDHD VYRDEALNNRFQIKGVELKS
                                  200                 220
Duck/Ukr/1/63                               WACQRGNIRCNIC I
NT/60/68               GYKDWILWISFAISCFLLCV VLLGFIMWACQRGNIRCNIC I
Aichi/2/68 (X-31)a     GYKDWILWISFAISCFLLCV VLLGFIMWACQRGNIRCNIC I
Aichi/2/68 (X-31)b     GYKDWILWISFAISCFLLCV VLLGFIMWACQRGNIRCNIC I
Mem/1/71                                        GNIR
Mem/102/72             GYKDWILWISFAISCFLLCV VLLGFIMWACQ‡GNIRCNIC I
Vic/3/75               GYKDWILWISFAISCFLLCV VLLGFIMWACQ‡GNIRCNIC I
Bang/79                GYKDWILWISFAISCFLLCV VLLGFIMWACQ‡GNIRCNIC I
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FIGURE 4. Amino acid sequence changes found between A/duck/Ukraine/1/63 and the human Hong Kong field strains isolated between 1968 and 1979. The data sources are duck/Ukraine/1/63 (37); NT/60/68 (20); Aichi/2/68 (X-31)[a] (21); Aichi/2/68 (X-31)[b] (11); Mem/1/71 (12,27); Eng/878/69, Qu/7/70 (20); Eng/42/72, PC/1/73 (12,23): Mem/102/72 (9,10,19); Vic/3/75 (22), Bang/79 (23). Peptide data for Vic/1/75 showed Asp not Asn at residue 53 in HA1 (12). The substitutions relative to Aichi/2/68 (X-31)[a] are shaded.

in the 1950's (52,53). This genetic identity makes its highly unlikely that the virus was preserved by sequential passage in some animal reservoir and possible alternative mechanisms have been suggested (29). The available evidence suggests that the main criteria governing the pandemic potential of any human influenza virus is the level of protective antibody present in the population. The unexpected re-emergence of the HI sub-type in 1977 emphasises this point and invalidates the suggestions that influenza pandemics are rigidly geared to a set order operating over a 70 year cycle (7,8,50,51,54,55).

V. REFERENCES

1. Webster, R.G., and Laver, W.G. in "The Influenza Viruses and Influenza" (E.D. Kilbourne, ed.), p.269. Academic Press, New York (1975).
2. Fazekas de St. Groth, S. in "The Influenza Virus Hemagglutinin" (W.G. Laver, H. Bachmayer and R. Weil, eds.), p.25. Springer-Verlag, Vienna (1978).
3. Laver, W.G., Downie, J.C., and Webster, R.G., Virology 59, 230 (1974).
4. Virelizier, J.L., Postlethwaite, R., Schild, G.C., and Allison, A.C., J. Expl. Med. 140, 1559 (1974).
5. Gerhard, W. in "The Influenza Virus Hemagglutinin" (W.G. Laver, H. Bachmayer and R. Weil, eds.), p.15. Springer-Verlag, Vienna (1978).
6. Russell, R.J., Burns, W.H., White, D.O., Anders, E.M., Ward, C.W., and Jackson, D.C., J. Immunol. 123, 825 (1979).
7. Fazekas de St. Groth, S., Arch. Environ. Hlth. 21, 293 (1970).
8. Fazekas de St. Groth, S., in "Negative Strand Viruses (B.W.J. Mahy and R.D. Barry, eds.), Vol. 2, p.741. Academic Press, London (1975).
9. Ward, C.W., and Dopheide, T.A., Virology 103, 37 (1980).
10. Dopheide, T.A., and Ward, C.W., J. Gen. Virol. 50, 329 (1980).
11. Ward, C.W., and Dopheide, T.A., Biochem. J. 193, 953 (1981).
12. Laver, W.G., Air, G.M., Dopheide, T.A., and Ward, C.W., Nature 283, 454 (1980).
13. Laver, W.G., Air, G.M., Webster, R.G., Gerhard, W., Ward, C.W., and Dopheide, T.A., Virology 98, 226 (1979).
14. Laver, W.G., Air, G.M., Webster, R.G., Gerhard, W., Ward, C.W., and Dopheide, T.A. in "Structure and Variation in Influenza Virus (Laver, W.G. and Air, G.M. eds.), p.295. Elsevier/North Holland, New York (1980).

15. Laver, W.G., Air, G.M., and Webster, R.G., J. Mol. Biol. 145, 339 (1981).
16. Moss, B.A., Underwood, P.A., Bender, V.J., and Whittaker, R.G. in "Structure and Variation in Influenza Virus" (Laver, W.G., and Air, G.M., eds.), p.329. Elsevier/North Holland, New York (1980).
17. Wilson, I.A., Skehel, J.J., and Wiley, D.C., Nature 289, 366 (1981).
18. Wiley, D.C., Wilson, I.A., and Skehel, J.J., Nature 289, 373 (1981).
19. Sleigh, M.J., Both, G.W., Brownlee, G.G., Bender, V.J., and Moss, B.A. in "Structure and Variation in Influenza Virus" (Laver, W.G., and Air, G.M., eds.), p.69. Elsevier/North Holland, New York (1980).
20. Sleigh, M.J., Both, G.W., Underwood, P.A., and Bender, V.J., J. Gen. Virol. In press.
21. Verhoeyen, M., Fang, R., Min-Jou, W., Devos, R., Huylebroeck, D., Saman, E., and Fiers, W., Nature 286, 771 (1980).
22. Min-Jou, W., Verhoeyen, M., Devos, R., Saman, E., Fang, R., Huylebroeck, D., and Fiers, W., Cell 19, 683 (1980).
23. Both, G.W., and Sleigh, M.J. This volume.
24. Ward, C.W., "Structure of Influenza Virus Hemagglutinin". Current Topics in Microbiology and Immunology. In Press.
25. Porter, A.G., Barber, C., Carey, N.H., Hallewell, R.A., Threlfall, G., and Emtage, J.S., Nature 282, 471 (1979).
26. Gething, M.J., Bye, J., Skehel, J.J., and Waterfield, M.D., Nature 287, 301 (1980).
27. Air, G.M., Blok, J., and Hall, R.M. in "Negative Strand Viruses" (D. Bishop and R.W. Compans, eds.), Elsevier, New York. In press.
28. Laver, W.G., and Webster, R.G., Virology 51, 383 (1973).
29. Laver, W.G., and Webster, R.G., Brit. Med. Bull., 35, 29 (1979).
30. Young, J.F., and Palese, P. Proc. Natl. Acad. Sci. USA 76, 6547 (1979).
31. Bean, W.J., Cox, N.J., and Kendal, A.P., Nature 284, 638 (1980).
32. Hinshaw, V.S., Bean, W.J., Webster, R.G., and Sriram, G. Virology 102, 412 (1980).
33. Scholtissek, C., Rohde, W., von Hoyningen, V., and Rott, R., Virology 87, 13 (1978).
34. Coleman, M.T., Dowdle, W.R., Pereira, H.G., Schild, G.C., and Chang, W.K., Lancet 2, 1384 (1968).
35. Scholtissek, C., Rohde, W., Harmes, E., and Rott, R., Virology 79, 330 (1977).
36. Ward, C.W., Webster, R.G., Inglis, A.S., and Dopheide, T.A., J. Gen. Virol. 53, 163 (1981).

37. Ward, C.W., and Dopheide, T.A., Biochem. J. 195, 337 (1981).
38. Ward, C.W., and Dopheide, T.A., Virology 95, 107 (1979).
39. Schild, G.C., Newman, R.W., Webster, R.G., Major, D., and Hinshaw, V.S. in "Structure and Variation in Influenza Virus" (Laver, W.G., and Air, G.M., eds.), p.373. Elsevier/North Holland, New York (1980).
40. Laver, W.G., Virology 45, 275 (1971).
41. Skehel, J.J., and Waterfield, M.D., Proc. Natl. Acad. Sci. USA. 72, 93 (1975).
42. Laver, W.G., and Webster, R.G., Virology 81, 482 (1977).
43. Ward, C.W., Gleeson, P.A., and Dopheide, T.A., Biochem. J. 189, 649 (1980).
44. Schacter, H., Narasimhan, S., and Wilson, J.R. in "Glycoconjugate Research" (J.D. Gregory and R.W. Jeanloz, eds.), p.575. Academic Press, New York, (1979).
45. Waterfield, M.D., Gething, M.J., Scrace, G., and Skehel, J.J. in "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds.), p.11. Elsevier/North Holland, New York, (1980).
46. Brown, L.E., Ward, C.W., and Jackson, D.C. In Preparation. (1981).
47. Sriram, G., Bean, W.J., Hinshaw, V.S., and Webster, R.G. Virology 105, 592 (1980).
48. Portner, A., Webster, R.G., and Bean, W.J., Virology 104, 235 (1980).
49. Webster, R.G., Yakhno, M., Hinshaw, V.S., Bean, W.J., and Murti, K.G., Virology 84, 268 (1978).
50. Davenport, F.M., Minuse, E., Hennessy, A.V., and Francis, T., Bull. Wld. Hlth. Org. 41, 453 (1969).
51. Masurel, N., Lancet 1, 907 (1969).
52. Nakajima, K., Desselberger, U., and Palese, P., Nature 274, 334 (1978).
53. Scholtissek, C., von Hoyningen, V., and Rott, R. Virology 89, 613 (1978).
54. Francis, T., Fed. Proc., 11, 808 (1952).
55. Fukumi, H., Bull. Wld. Hlth. Org. 41, 469 (1969).
56. Bucher, D.J., Li, S.S-L., Kehoe, J.M., and Kilbourne, E.D., Proc. Natl. Acad. Sci. USA. 78, 238 (1976).
57. Waterfield, M.D., Gething, M.J., Scrace, G., Skehel, J.J., Brit. Med. Bull. 35, 57 (1979).
58. Klenk, H.D. in "Structure and Variation in Influenza Virus" (W.G. Laver and G.M. Air, eds.), p.213. Elsevier/North Holland, New York, (1980).

THE EXTENT OF HAEMAGGLUTININ VARIATION DURING ANTIGENIC DRIFT IN THE HONG KONG SUBTYPE OF INFLUENZA FROM 1968 TO 1979

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ABSTRACT

We have determined the cDNA sequences for the haemagglutinin genes from five strains of the H3 (Hong Kong) subtype of influenza by a combination of gene cloning and primed cDNA synthesis by reverse transcriptase using the HA genome RNA segment as a template. The amino acid sequences predicted from these data allow us to examine the extent and location of amino acid variation in the haemagglutinin protein occurring between 1968 and 1979. There are four areas of variable sequence in the protein, three of which correlate with the position of amino acid changes in antigenically distinct variant strains selected in the laboratory. The information allows us to predict areas of the protein likely to be involved in antibody binding. These regions correlate well with the antigenic sites predicted from the three-dimensional structure of the haemagglutinin (5,17). The potential for further antigenic drift within the H3 subtype is discussed.

INTRODUCTION

The occurrence of antigenic variation among influenza viruses has been shown to be due to two phenomena, antigenic shift and antigenic drift. The former involves the acquisition by a virus of one or two genes coding for the antigenic surface proteins, haemagglutinin (HA) and neuraminidase (NA). For example, the shift from the Asian to the Hong Kong subtype of influenza in 1968 was characterized by the appearance of virus strains carrying the H3-type HA and the N2-type NA; strains of the Asian subtype had been of the H2N2 type.

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Antigenic drift is the result of variation within a viral subtype as the virus evolves under the selective pressure provided by host immunity. Our work has been concerned with analysing variation in the viral HA during antigenic drift in the Hong Kong subtype, which first arose in humans in 1968 and is still circulating in the population. We reasoned that areas of the HA protein might remain unchanged during antigenic drift so that the biological and functional integrity of the molecule would be preserved. In contrast, because of the immune selective pressure under which the virus is evolving, we expected to find regions in the HA protein which had undergone amino acid substitution in order to disrupt antibody binding.

To test these ideas, we have determined partial or complete nucleotide sequences for the HA genes from several viral strains within the H3 subtype, isolated in 1968, 1969, 1970, 1972 and 1979. The HA amino acid sequences thus predicted for these strains have also been compared with the amino acid sequence predicted for a 1975 strain (1). By comparing regions of amino acid variation with the location of amino acid substitutions in the HA of antigenic variants selected with monoclonal antibodies (2,3), or a fraction of whole serum (4), we can predict the location of some antigenically important sequences in the HA protein. These sequences can be located on the three-dimensional structure of the 1968 HA protein determined recently (5). The potential for further antigenic drift within the influenza H3 subtype is discussed.

MATERIALS AND METHODS

Virus purification and preparation of viral RNA

Methods for the growth and purification of virus and preparation of viral RNA have been described previously (6). Influenza strains A/Eng/878/69 (Eng69), A/Qu/7/70 (Qu70) and A/Mem/102/72 (a PR8 recombinant (Mem72)) were obtained from Dr. W.G. Laver. Strain A/NT/60/68 (NT68) was obtained from Dr. S. Fazekas de St. Groth. A/BK/1/79 (BK79) was provided by Dr. R. Webster.

Cloning and sequencing of HA gene copies

Methods for making DNA copies of the HA genes from the strains Mem72 and NT/60/68/29C (4) and the cloning of these copies in pBR322/ E. coli RR1 have been outlined previously (7). The gene copies were sequenced by the method of Maxam and Gilbert (8) as described (7).

Determination of the HA sequences from viral RNA

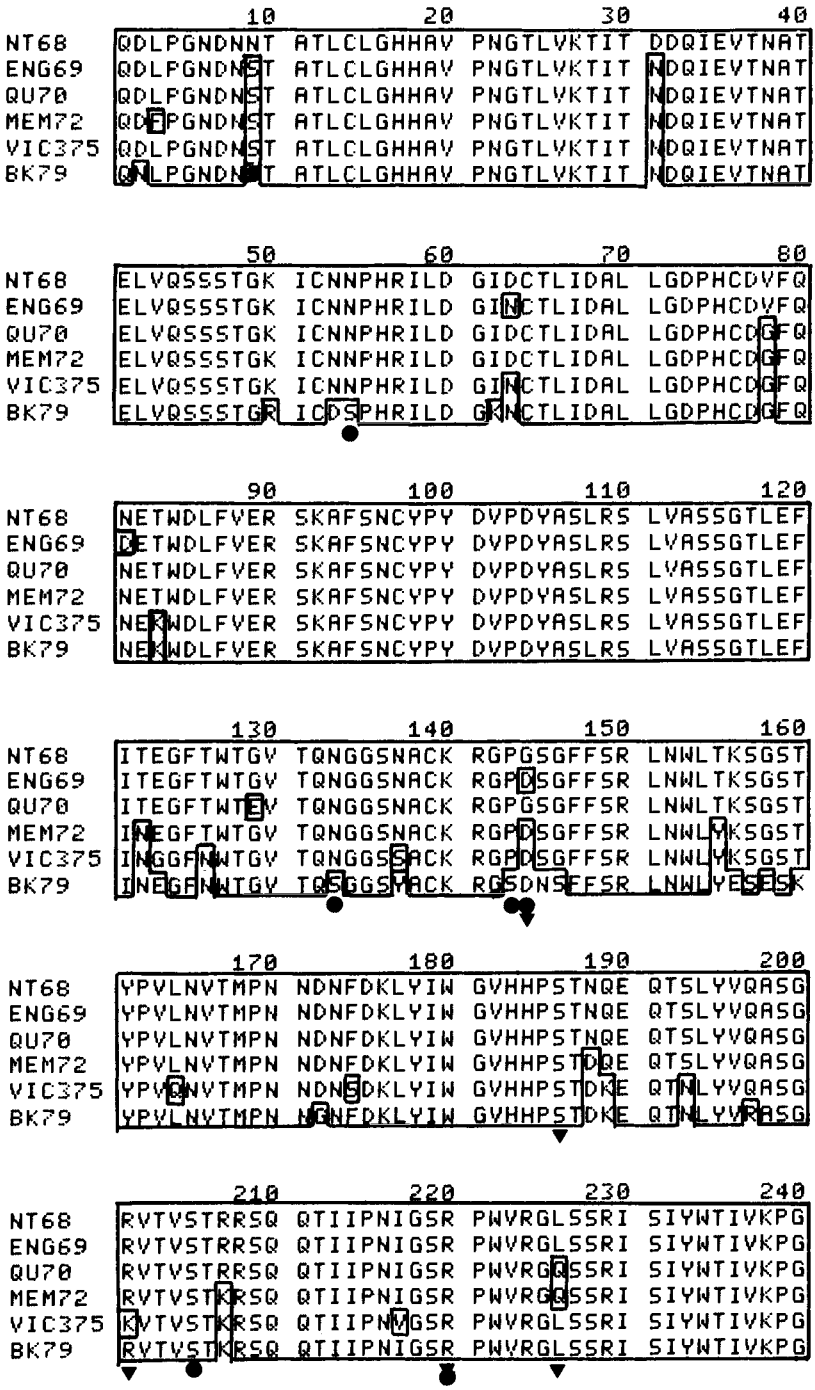
Since the viral populations with which we are dealing are mixed (2,3,9,10,11), gene sequences determined indirectly from the viral RNA are likely to be more representative than those determined from single cloned gene copies. Thus, for comparative sequencing required for this evolutionary study, we prepared small DNA fragments (27-150 base pairs) from the cloned dsDNA copy of the HA genes from strains Mem72 and 29C (6,7) by digestion with appropriate restriction enzyme(s). These were isolated by polyacrylamide gel electrophoresis (10). The DNA fragment was mixed with total influenza genome RNA from a particular strain, heated to 95^{o}C for 1 min and chilled. The (+) strand of the fragment anneals to its complementary sequence in the (-) stranded HA gene RNA segment and can act as a primer for reverse transcriptase. Thus, cDNA can be synthesised and size related copies are produced by incorporating dideoxynucleoside triphosphates in the reactions (12). The sequence can be deduced by resolving the cDNA products on a polyacrylamide gel (13).

RESULTS AND DISCUSSION

Restriction fragment-primed copying of vRNA was used to generate sequence data for the HA genes of a series of H3 field strains, as described in Methods. The nucleotide sequences obtained in this way have been published previously (7,10,13,14). The sequence we now report for the HA gene of Mem72 is the same as NT68 at bases 110, 127 and 327, in contrast with a previous report (7). Figure 1 summarises the amino acid sequences predicted from the gene sequence data for the HA1 region of the mature HA for the H3 strains NT68, Eng69, Qu70, Mem72 and BK79. Also included in the figure is data for the HA of the strain Vic375, determined by Min Jou *et al.* (1).

Conserved areas in the HA of H3 subtype viruses

From the results summarised in Fig. 1 it is apparent that there are two blocks of conserved amino acids in the HA1 subunit of H3 influenza viruses. These blocks include residues 84-121, and residues 279-328 at the C-terminus of HA1. The second of these regions falls within the "stalk" structure supporting the globular region of HA1 at the top of the HA spike (5). The smaller HA subunit, HA2, also forms part of this stalk structure. Only 3 amino acid changes are seen in HA2 between NT68 and BK79 (13) and only one of these (glu→gly) at HA2 residue 150 is non-conservative. Since the HA2 subunit is also relatively well conserved between viral subtypes (9,15)


10 20 30 40
NT68 QDLPGNDNNT ATLCLGHHAV PNGTLVKTIT DDQIEVTNAT
ENG69 QDLPGNDNST ATLCLGHHAV PNGTLVKTIT NDQIEVTNAT
QU70 QDLPGNDNST ATLCLGHHAV PNGTLVKTIT NDQIEVTNAT
MEM72 QDLPGNDNST ATLCLGHHAV PNGTLVKTIT NDQIEVTNAT
VIC375 QDLPGNDNST ATLCLGHHAV PNGTLVKTIT NDQIEVTNAT
BK79 QNLPGNDNST ATLCLGHHAV PNGTLVKTIT NDQIEVTNAT
50 60 70 80
NT68 ELVQSSSTGK ICNNPHRILD GIDCTLIDAL LGDPHCDVFQ
ENG69 ELVQSSSTGK ICNNPHRILD GINCTLIDAL LGDPHCDVFQ
QU70 ELVQSSSTGK ICNNPHRILD GIDCTLIDAL LGDPHCDGFQ
MEM72 ELVQSSSTGK ICNNPHRILD GIDCTLIDAL LGDPHCDGFQ
VIC375 ELVQSSSTGK ICNNPHRILD GINCTLIDAL LGDPHCDGFQ
BK79 ELVQSSSTGR ICDSPHRILD GKNCTLIDAL LGDPHCDGFQ
90 100 110 120
NT68 NETWDLFVER SKAFSNCYPY DVPDYASLRS LVASSGTLEF
ENG69 DETWDLFVER SKAFSNCYPY DVPDYASLRS LVASSGTLEF
QU70 NETWDLFVER SKAFSNCYPY DVPDYASLRS LVASSGTLEF
MEM72 NETWDLFVER SKAFSNCYPY DVPDYASLRS LVASSGTLEF
VIC375 NEKWDLFVER SKAFSNCYPY DVPDYASLRS LVASSGTLEF
BK79 NEKWDLFVER SKAFSNCYPY DVPDYASLRS LVASSGTLEF
130 140 150 160
NT68 ITEGFTWTGV TQNGGSNACK RGPGSGFFSR LNWLTKSGST
ENG69 ITEGFTWTGV TQNGGSNACK RGPDSGFFSR LNWLTKSGST
QU70 ITEGFTWTEV TQNGGSNACK RGPGSGFFSR LNWLTKSGST
MEM72 INEGFTWTGV TQNGGSNACK RGPDSGFFSR LNWLMKSGST
VIC375 INGGFNWTGV TQNGGSSACK RGPDSGFFSR LNWLYKSGST
BK79 INEGFNWTGV TQSGGSMACK RGSDNSFFSR LNWLYESESK
170 180 190 200
NT68 YPVLNVTMPN NDNFDKLYIW GVHHPSTNQE QTSLYVQASG
ENG69 YPVLNVTMPN NDNFDKLYIW GVHHPSTNQE QTSLYVQASG
QU70 YPVLNVTMPN NDNFDKLYIW GVHHPSTNQE QTSLYVQASG
MEM72 YPVLNVTMPN NDNFDKLYIW GVHHPSTDQE QTSLYVQASG
VIC375 YPVQNVTMPN NDNSDKLYIW GVHHPSTDKE QTNLYVQASG
BK79 YPVLNVTMPN NGNFDKLYIW GVHHPSTDKE QTNLYVRASG
210 220 230 240
NT68 RVTVSTRRSQ QTIIPNIGSR PWVRGLSSRI SIYWTIVKPG
ENG69 RVTVSTRRSQ QTIIPNIGSR PWVRGLSSRI SIYWTIVKPG
QU70 RVTVSTRRSQ QTIIPNIGSR PWVRGQSSRI SIYWTIVKPG
MEM72 RVTVSTKRSQ QTIIPNIGSR PWVRGQSSRI SIYWTIVKPG
VIC375 KVTVSTKRSQ QTIIPNMGSR PWVRGLSSRI SIYWTIVKPG
BK79 RVTVSTKRSQ QTIIPNIGSR PWVRGLSSRI SIYWTIVKPG

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          250        260        270        280
NT68   DVLVINSNGN LIAPRGYFKM RTGKSSIMRS DAPIDTCISE
ENG69  DVLVINSNGN LIAPRGYFKM RTGKSSIMRS DAPIDTCISE
QU70   DVLVINSNGN LIAPRGYFKM RTGKSSIMRS DAPIDTCISE
MEM72  DILVINSNGN LIAPRGYFKM RTGKSSIMRS DAPIGTCISE
VIC375 DILVINSNGN LIAPRGYFKM RTGKSSIMRS DAPIGTCSSE
BK79   DILIINSNGN LIAPRGYFKI RTGKSSIMRS DAPIGTCSSE

          290        300        310        320
NT68   CITPNGSIPN DKPFQNVNKI TYGACPKYVK QNTLKLATGM
ENG69  CITPNGSIPN DKPFQNVNKI TYGACPKYVK QNTLKLATGM
QU70   CITPNGSIPN DKPFQNVNKI TYGACPKYVK QNTLKLATGM
MEM72  CITPNGSIPN DKPFQNVNKI TYGACPKYVK QNTLKLATGM
VIC375 CITPNGSIPN DKPFQNVNKI TYGACPKYVK QNTLKIATGM
BK79   CITPNGSIPN DKP-QNVNKI TYGACPKYVK QNTLKLATGM

          330
NT68   RNVPEKQT
ENG69  RNVPEKQT
QU70   RNVPEKQT
MEM72  RNVPEKQT
VIC375 RNVPEKQT
BK79   RNVPEKQT
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Figure 1. Comparison of amino acid sequences in the HA1 regions of strains from the H3 subtype. The amino acid data for NT68, Eng69 and Qu70 was obtained earlier (10). The Mem72 and Vic375 (A/Vic/3/75) sequences are derived from cloned gene copies (1,7) but the Mem72 sequence is also supported by protein sequence data (21). The extra asn residue between amino acids 8 and 9 is omitted from Vic/375 for clarity (1). Residues altered in later strains compared with NT68 are boxed. The sites of amino acid changes in laboratory selected variants are indicated. (●) Variants selected with monoclonal antibodies; (▼) variants selected with a fraction of whole serum. The one letter code for amino acids is A(Ala), R(Arg), N(Asn), D(Asp), C(Cys), Q(Gln), E(Glu), G(Gly), H(His), I(Ile), L(Leu), K(Lys), M(Met), F(Phe), P(Pro), S(Ser), T(Thr), W(Trp), Y(Tyr) and V(Val).

it is evident that conservation of the stalk region of the HA is vital to preserve HA structure and function.

The second conserved region seen in the HA1 of H3 influenza strains includes residues generally located in the upper globular region of the HA molecule (5). Of the residues 84-121, several are conserved between viral subtypes (9,15) and may form a part of the HA active site containing the cell receptor (5). Iodination of whole virus results in modification of a tyrosine in this region (tyrosines occur at residues 98, 100 and 105 - Fig. 1) and iodination at this site results in disruption of the haemagglutinating activity of the virus (16). Therefore at least some of the residues between 84 and 121 may be conserved in order to maintain HA function. Interestingly, the frequency of silent base changes in this region of the HA gene seems to be lower than is seen elsewhere in the gene (13). Perhaps constraints on HA gene evolution are imposed not only by the effect of resulting amino acid changes in the HA protein, but also by a need to conserve sequence in regions of the gene involved in RNA folding or protein recognition.

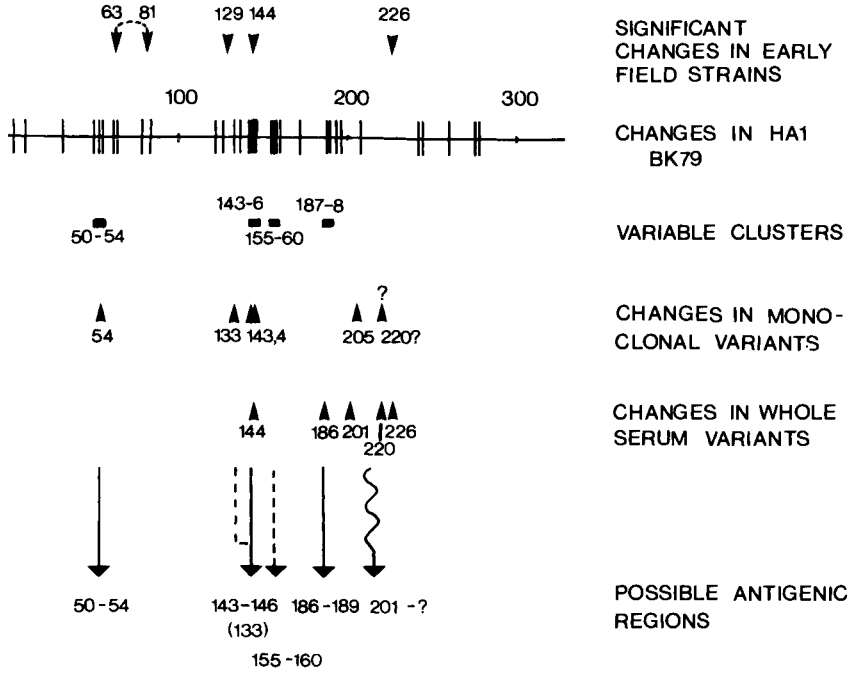

Figure 2. Summary of the information used to predict the antigenically important regions of the HA protein for the H3 subtype.

Variation in the HA during H3 subtype evolution

Almost all of the HA amino acid sequence differences between a 1968 H3 strain and later isolates of the subtype occur in that part of the HA1 making up the globular region at the top of the HA spike and in the region hinging this structure to its supporting stalk (5). These are the areas of the protein which are most accessible and are most likely to be involved in antibody binding (17).

Amino acid changes are not distributed evenly over the HA1 but are concentrated within particular regions (Fig. 1). In some places, clusters of variable amino acids are seen with changes accumulating in adjacent amino acids as the protein evolves during subtype evolution. Some clusters of amino acids have been noted by Laver *et al.* (18) who compared partial amino acid sequence data for HA's from several field isolates of the H3 subtype. We are now able to extend this observation to other regions and to identify four regions in the HA1 subunit where such clusters of altered amino acids occur (Fig. 1 and 2).

Identification of antigenically important amino acid residues

Antigenic sites may be made up of a group (5-6) of amino acids arranged sequentially on the protein (continuous site), or may be discontinuous, involving amino acids from distant parts of the molecule brought together by protein folding (19, 20). Where antigenic sites are of the continuous type, progressive antigenic drift may lead to clustering of amino acid changes, described above. Thus, these sites may be recognised by the accumulation of adjacent amino acid changes as the subtype evolves.

Identifying amino acid changes which fall within discontinuous antigenic sites and distinguishing them from isolated amino acid changes which are antigenically unimportant is clearly much more difficult. For this reason it is necessary to use independent methods for identifying the antigenic consequences of particular HA1 amino acid changes. One way in which this has been achieved is by selection in the laboratory of variants able to grow in the presence of neutralising antibody. Selection of variants in this way has been carried out using monoclonal antibodies (2,3) or a subfraction of whole antiserum (4). Amino acids altered in these antigenically distinct variants have been identified by analysis of altered HA peptides (2,11,18) or by sequencing of variant HA genes (Both and Sleigh, unpublished results). Amino acids altered in these variants are identified in Figures 1 and 2. Figure

2 also summarises the positions where clusters of altered amino acids are seen in H3 field strains.

An alternative approach to the identification of antigenically important amino acid changes has involved the titration of field strains isolated early in H3 subtype evolution with a panel of monoclonal antibodies directed against a 1968 strain (10). These early isolates contain few amino acid changes, some of which are also found in laboratory variants (10,11). In this way it has been possible to identify as antigenically important the amino acid changes at residues 144 and either 63 or 81 in Eng69 and changes at residues 129 and 226 in Qu70 (10).

Prediction of the antigenically important regions of HA

Figure 2 summarises information on clusters of variable amino acids in the HA1 protein of the H3 subtype viruses, together with the sites of antigenically important amino acid changes in early field strains and in laboratory selected variants. By using all the available information we are able to predict regions of the HA1 that are likely to be involved in antibody binding. These are also summarised in Figure 2. The region 50-54 contains three altered amino acids in BK79 and residue 54 is the site of a change in one variant selected with a monoclonal antibody (2). Four residues in the region 143-146 are altered in BK79 and in some earlier field strains (18) and antigenically important changes are also seen in this region in the early field strains (10), monoclonally selected variants (2) and variants selected with a fraction of whole serum (11). A cluster of altered amino acids including residues 188 and 189 in BK79 is supported by a variant whose amino acid change maps at residue 186 (11; Both and Sleigh, unpublished results).

In addition to those regions which may be associated with continuous antigenic sites on the HA protein, some evidence has accumulated that residues more scattered on the molecule may contribute to antigenicity, perhaps forming one or more discontinuous sites. Several of the laboratory-selected variants and an antigenically-important change in one of the field strains (at residue 226) map in the C-terminal half of HA1, in a region where very few changes are seen in H3 field strains, including BK79 (Fig. 2). In addition, changes at residues 129, and 63 or 81 are seen to be important in early field isolates, although these changes are not closely associated with clusters of variable amino acids (10). Residue 133, altered in BK79, must be associated with the same antigenic site containing residue 143 since the same monoclonal antibody is able to recognise changes at both residues (2,3).

Location of predicted antibody binding sites on the HA spike

Since the three-dimensional arrangement of an H3 HA molecule has now been determined (5), it is possible to locate on the HA spike, the sites predicted to be antigenically-important. This is shown in Fig. 3, where amino acid changes seen in the HA1 of BK79 are marked with black circles, and the sites of predicted antigenic importance are indicated. It can now be seen that the four variable clusters occur at the protein surface, corresponding to the antigenic sites A, B and C proposed by Wiley *et al.* (17). Some changes from other parts of the HA sequence are associated by protein folding with these four sites, e.g. residue 133 with site A and residues 275 and 278 with the 50-54 variable cluster (site C). Residue 129 lies on the surface between sites A and B.

A cluster of four altered amino acids includes residues 63, 81 and 83 (Fig. 3). The changes at positions 81 and 83 cause the loss of a glycosylation site, while the one at residue 63 creates a new potential site for carbohydrate binding. If the new site is, in fact, glycosylated, then only a small shift in the position of the carbohydrate would be expected (Wiley, personal communication). However, the results with Eng69 (10) suggest that either the shift of the carbohydrate, or its disappearance, can affect HA antigenicity.

Very few changes in epidemic field isolates map in the interface region between monomers of the HA spike (Fig. 3). Several antigenic variants have amino acid changes mapping in this region, and on this basis Wiley *et al.* proposed the existence of an antigenic site D near the interface (17). The changes found in laboratory variants mapping in site D were generally non-conservative (e.g. arg→gly at residue 201, arg→ile at residue 220 (11,14) ser→tyr at 205 (2). Several more variants selected with monoclonal antibodies have now been selected with changes mapping in site D (Webster *et al.*, this volume) and both these and the above site D variants are considerably reduced in their ability to grow (Webster, personal communication; Underwood, personal communication). Thus it seems likely that while non-conservative amino acid changes in the D region can successfully disrupt antibody binding, they may do so at the expense of virus survival. Therefore, variants with significant changes in this region may not be successful in the field. The lack of dramatic amino acid changes in this region in the major epidemic strains is consistent with this idea.

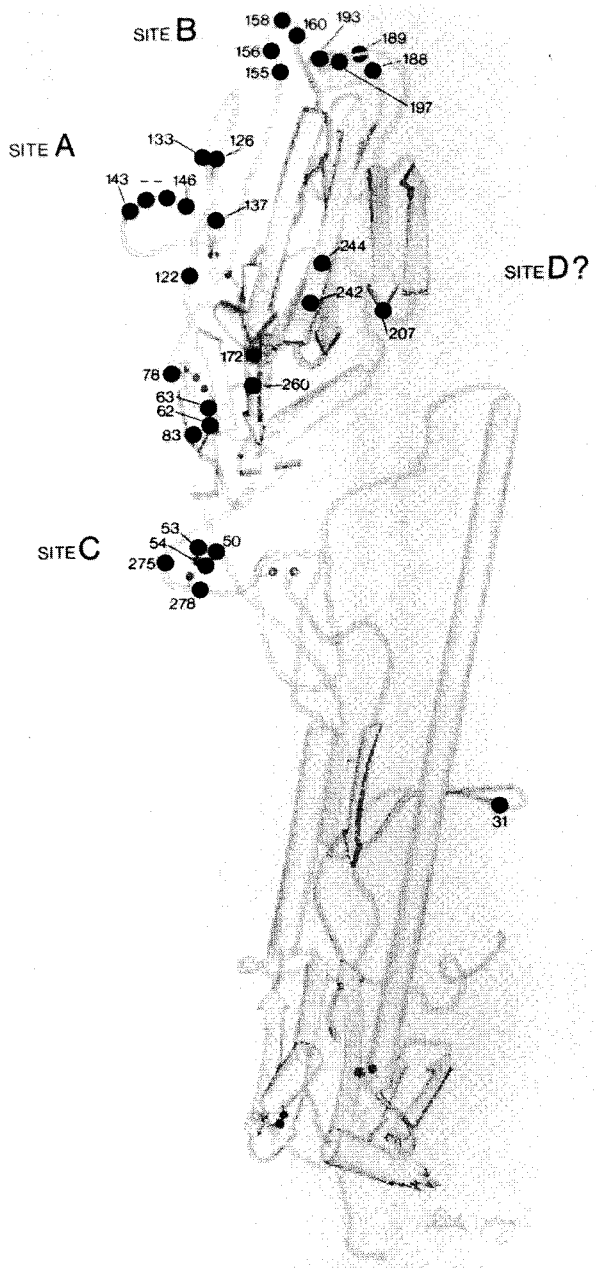

Figure 3. Schematic drawing of the monomer of the HA from Aichi 68 (5,17) showing the locations of amino acid changes in HA1 for BK79. The location of predicted antigenic regions is indicated in the figure and text.

Future evolution of the H3 subtype

The accumulation of changes in the HA gene and protein has followed a roughly linear path since the H3 subtype first appeared in man in 1968 (Both and Sleigh, manuscript in preparation). By 1979 approximately 10% of the amino acids in HA1 have changed (Fig. 1). It has been proposed that amino acids involved in antibody binding are anchored in the HA spike in a framework of conserved amino acids (5,17) and only one of these proposed anchor residues has changed in BK79 (residue 50, which has undergone a conservative change from lysine to arginine). Of the amino acids thought to form the antigenic regions A, B and C, relatively few remain unchanged as the HA has evolved from 1968 to 1979. Scope for further antigenic change in the HA of the H3 subtype may thus be limited. However, such a judgement is based on superimposing amino acid changes on an HA three-dimensional structure determined for a 1968 virus (5). The accumulation of amino acid changes over several years may have considerably altered the topography of residues at the protein surface, reducing the potential role of particular amino acids in antibody binding, and bringing into prominence other amino acids not previously thought to be antigenically important. In addition, we have not yet seen an example of a residue which has undergone more than one change, although a consideration of the codons for residues such as 144 suggest that potential exists for further disruptive changes at these positions. Providing that further changes can occur without seriously affecting the ability of the virus to grow (e.g. dramatic changes in the site D region may be prohibited) then it seems that some potential still exists for the emergence of new antigenic variants among H3 subtype isolates.

Finally, the epidemiological significance of amino acid changes in the HA is not completely understood. It must also be considered that the success of epidemic strains within a subtype may be attributable to mutations in other genes of the virus occurring in concert with those in the HA gene.

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REFERENCES

1. Min Jou, W., Verhoeyen, M., Devos, R., Saman, E., Fang, R., Huylebroeck D., Fiers, W., Threlfall, G., Barber, C., Carey N. and Emtage, S. (1980). *Cell 19*, 683.
2. Laver, W.G., Air, G.M., Webster, R.G., Gerhard, W., Ward, C.W. and Dopheide, T.A.A. (1979). *Virology 98*, 226.
3. Webster, R.G. and Laver, W.G. (1980). *Virology 104*, 139.
4. Fazekas de St. Groth, S. and Hannoun, C. (1973). *C.R. Acad. Sci. Paris Ser. D. 276*, 1917.
5. Wilson, I.A. , Skehel, J.J. and Wiley, D.C. (1981). *Nature 289*, 366.
6. Sleigh, M.J., Both, G.W. and Brownlee, G.G. (1979). *Nucleic Acid Res. 6*, 1309.
7. Sleigh, M.J., Both, G.W., Brownlee, G.G., Bender, V.J. and Moss, B.A. (1980). *Structure and Variation in Influenza virus (eds. Laver, W.G. and Air, G.M.)* 69 (Elsevier, Amsterdam).
8. Maxam, A.M. and Gilbert, W. (1977). *Proc. Nat. Acad. Sci. USA. 74*, 560.
9. Both, G.W. and Sleigh, M.J. (1980). *Nucleic Acids Res. 8*, 2561.
10. Sleigh, M.J., Both, G.W., Underwood, P.A. and Bender, V.J. (1981). *J. Virol.* In press.
11. Moss, B.A., Underwood, P.A., Bender, V.J. and Whittaker, R.G. (1980). *Structure and Variation in Influenza Virus (eds. Laver, W.G. and Air, G.M.)* 329. (Elsevier, Amsterdam).
12. Sanger, F., Nicklen, S. and Coulson, A.R. (1977). *Proc. Natl. Acad. Sci., USA 74*, 5463.
13. Both, G.W. and Sleigh, M.J. (1981). Submitted to *J. Virology.*
14. Both, G.W., Sleigh, M.J., Bender, V.J. and Moss, B.A. (1980). *Structure and Variation in Influenza Virus. (eds. Laver, W.G. and Air, G.M.).* 81 *(Elsevier, Amsterdam).*
15. Gething, M.J., Bye, J., Skehel, J. and Waterfield, M. (1980). *Nature 287*, 301.
16. Bender, V.J., Underwood, P.A. and Moss, B.A. unpublished results.
17. Wiley, D.C., Wilson, I.A. and Skehel, J.J. (1981). *Nature 289*, 373.
18. Laver, W.G., Air, G.M., Dopheide, T.A. and Ward, C.W. (1980). *Nature 283*, 454.

19. Atassi, M.Z. (1977). *Immunochemistry of Proteins. (ed. Atassi, M.Z.) 2*, 77 (Plenum, New York).
20. Atassi, M.Z. (1978). *Immunochemistry 15*, 909.
21. Ward, C.W. and Dopheide, T.A.A. (1980). *Virology 103*, 37.

IMMUNOCHEMICAL PROPERTIES OF INFLUENZA VIRUS HEMAGGLUTININ AND ITS FRAGMENTS[1]

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ABSTRACT

Several immunochemical approaches have been applied to the characterisation of the antigenic determinants on the hemagglutinin (HA) of influenza virus. Using various types of radioimmunoassay it has been shown that antigenic activity is carried by the N-terminal 168 amino acid residues of the "heavy chain", HA_1 (HA_1CN1) and by the C-terminal 90 amino acid residues of the "light chain", HA_2 (HA_2CN1). One other cyanogen bromide fragment HA_1CN3 is bound by antibodies raised against isolated HA but not by antibodies raised against intact virus, suggesting that this region is located in an inaccessible region of the HA spike when present *in situ* on the virus.

Competitive binding of radiolabeled monoclonal antibodies has provided some insight into the topography of distinct antigenic regions on the surface of the HA molecule. Monoclones recognising epitopes in the same antigenic site reciprocally block one another's attachment to HA, whereas others bind non-competitively to separate sites on the molecule. At least four antigenic sites have been defined in this way.

Some monoclonal antibodies that bind well to virions and to HA fail to bind to HA_1 or HA_2. Some degree of

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enhancement of binding to HA can be demonstrated in the presence of certain combinations of two different monoclones.

The "loop" formed by amino acids 140-150 within HA_1CN1 has been synthesised and then polymerised using glutaraldehyde. When tested against anti-viral IgG in a radioimmunoassay, this preparation was shown to possess antigenic activity.

INTRODUCTION

The antigenic properties of proteins can be completely mapped only by the application of a variety of techniques. Chemical modification of specific amino acid residues, cleavage of the peptide bond at different locations giving rise to a number of fragments, and the synthesis of particular regions of the molecule under study have been the techniques favoured for the production of antigen derivatives which can be individually studied for their immunochemical properties. In the two or three instances where the antigenic structures of proteins have been reported to be completed, the structure of the molecule determined by x-ray analysis has also been available.

More recently, hybridoma technology has been extensively used to develop additional techniques which were until now not possible using normal, i.e. polyclonal antisera. In the case of influenza virus hemagglutinin, each of the above techniques has been applied and from the evidence a picture is emerging of the immunochemical structure of this antigen.

An additional feature of hemagglutinin is that, being a glycoprotein, containing a number of distinct oligosaccharide side-chains, it allows an examination to be made of the antigenic properties of these individual carbohydrates, an aspect which has been largely neglected in the field of immunochemistry.

In this paper a summary of our studies of the antigenic and immunogenic properties of polypeptide and carbohydrate fragments derived from the hemagglutinin of A/Mem/102/72 is presented together with results on the use of monoclonal antibodies and synthetic peptides to further characterise the antigenic structure of HA.

METHODS

The viruses used were A/Bellamy/42(HON1) and the "recombinants" derived by genetic reassortment with the

hemagglutinins A/Memphis/102/72(H3) and A/Aichi/2/68(H3). These viruses are referred to as Bel,Mem_H-Bel_N and X-31 respectively and were grown in the allantoic cavity of 10-day embryonated chicken eggs. Hemagglutinin was prepared from purified viruses by electrophoresis on cellulose acetate blocks and HA_1 and HA_2 chains were separated by the method of Laver (1). Fragments of HA and HA_1 were prepared by cyanogen bromide (CNBr) cleavage and isolated according to Jackson et al (2,3). Radioiodination was carried out by a modification (4) of the chloramine T method and tritiation as previously described (5). Antisera to purified viruses were raised by inoculation, with Freund's complete adjuvant, into outbred New Zealand rabbits. IgG was prepared from these antisera by affinity chromatography on protein A-Sepharose CL-4B and used either untreated or after adsorption to remove antibodies directed against carbohydrate (2,3,6,7). Antigen-antibody complexes were detected either by use of protein A-Sepharose as a solid phase immunoadsorbent in cases where antigen was radiolabeled (6), or by a solid phase method in the case of non-radiolabeled antigen (5). The monoclonal antibodies against X-31 virus used in this study were a generous gift from Dr. Walter Gerhard of the Wistar Institute.

RESULTS

Antigenicity of CNBr-Derived Peptides

Purified HA or HA_1 from influenza A/Mem/102/72(H3) virus was cleaved by CNBr to produce the fragments depicted in Fig. 1.

Each CNBr fragment was radiolabeled and tested for antigenic activity against IgG from the sera of rabbits immunized with the purified virus (Table 1).

Antigenic activity was detected by this particular approach only in those preparations containing the fragment HA_1CN1, which represents the N-terminal 168 amino acid residues of HA_1. Titration of anti-viral IgG against equimolar amounts of each fragment revealed that HA_1 displays the highest antigenic activity, followed by P1, which contains two of the largest CNBr fragments from HA_1 and the largest from HA_2 linked together by disulfide bonds. The large size and preservation of S-S bonds would tend to maintain conformational integrity within P1. The isolated peptide HA_1CN1 itself was also antigenic despite the loss of conformation inevitably resulting from its separation from structurally important regions remote from residues 1-168 and

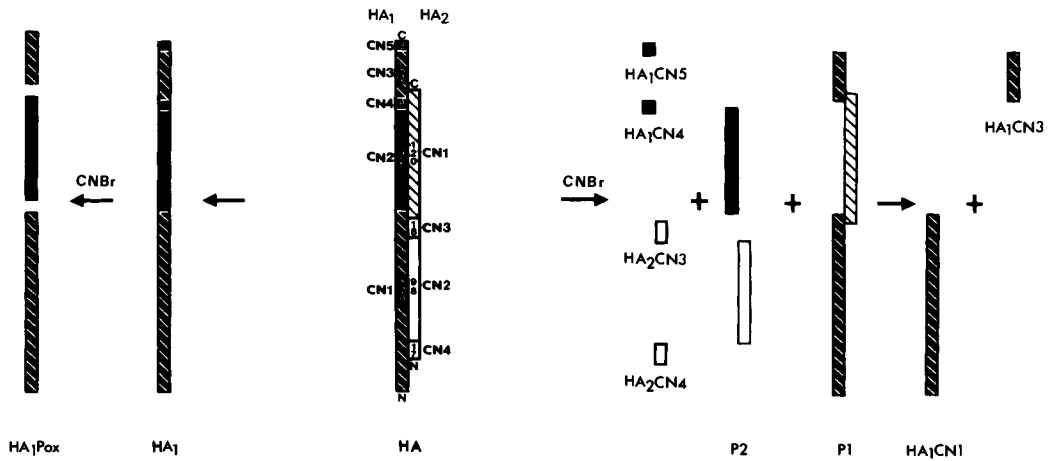

FIGURE 1. Schematic representation of the hemagglutinin monomer of influenza virus A/Memphis/102/72 and its cyanogen bromide cleavage products. HA_1 and fragments derived from it are represented by solid bars; HA_2 and its fragments by open bars. Cross-hatching indicates fragments containing carbohydrate side-chains. These fragments also contain half cystine residues. Cyanogen bromide cleavage of HA_1 yields a mixture of three major fragments in oxidised form (HA_1Pox). Cleavage of whole HA monomer gives rise to P2, a mixture of HA_1CN2 and HA_2CN2; P1 which is a disulfide-linked complex of HA_1CN1, HA_1CN3 and HA_2CN1 and the octapeptides from HA_1 together with the seventeen and eighteen-residue peptides from HA_2, viz HA_1CN4, HA_1CN5, HA_2CN4 and HA_2CN3 respectively. (Modified from Jackson et al. 1979 J.Immunol. 123 with permission).

TABLE 1. Binding of IgG to Radiolabeled Hemagglutinin Fragments[a]

IgG	Radiolabeled fragment						
	$MemHA_1$	P1	HA_1Pox	P2	HA_1CN1	HA_1CN3	HA_1CN4 HA_1CN5 HA_2CN3 HA_2CN4
Anti-(Mem_H-Bel_N)							
Unadsorbed	38[b]	44	27	8	20	4	0
Adsorbed[c]	31	31	17	3	13	3	0
Anti-Bel							
Unadsorbed	32	40	24	2	15	4	0
Adsorbed	7	4	3	2	2	2	0
Normal rabbit	3	2	2	3	1	1	0

[a]Five micrograms of IgG were incubated with 0.25 pmoles radiolabeled hemagglutinin fragment in a total volume of 20 ul for 3 hr at room temperature. Antigen-antibody complexes were detected by the addition of 50 ul of a 20% suspension of protein A-Sepharose.
[b]Results are expressed as the percentage of the radiolabeled fragment that is bound by IgG.
[c]IgG was adsorbed with influenza virus A/shearwater/E.Aust./1/72(H6) to remove antibody directed against oligosaccharide side-chains.

from reduction and subsequent re-oxidation of disulfide bonds.

Alteration of a particular residue or residues can cause far-reaching effects on the overall structure of proteins, consequently an absence of activity need not mean that the antigenic site of a particular derivative has been directly affected. To avoid any problems associated with the modification of tyrosine, histidine and possibly methionine residues effected by chloramine T induced radioiodination an assay was developed (5) in which the fragments were not radiolabeled. The antigen, adsorbed to wells of polyvinyl plates, was exposed to rabbit IgG, then washed, and bound antibody detected by ^{125}I-protein A or radioiodinated rabbit IgG directed against mouse immunoglobulin. This sensitive assay revealed that P2, which comprises HA_1CN2 plus HA_2CN2 possesses slight but significant activity(5).

Although insoluble, HA_2 can be sonicated into solution and subsequently radioiodinated. This preparation fails however to be bound by anti-viral antibody. This result may indicate that either: (i) HA_2 is not antigenic (ii) HA_2 is not capable of eliciting antibody formation when virus is inoculated into animals or (iii) insertion of an iodine atom into tyrosine or histidine residues destroys the antigenicity of HA_2. In fact, the last possibility seems to be the case because non-labeled HA_2 adsorbed to polyvinyl wells is capable of binding anti-viral IgG (Fig. 2) although

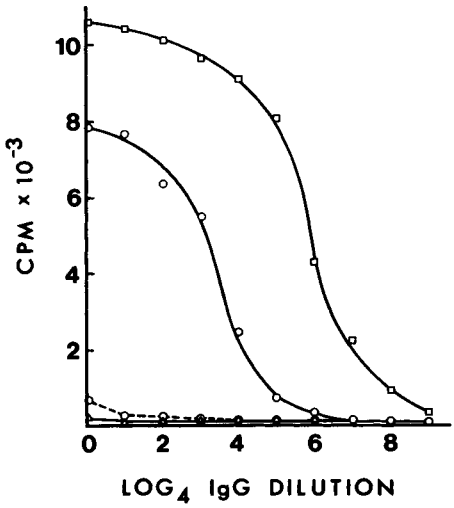

FIGURE 2. Direct binding of influenza A/shearwater/E.Aust./1/72(H6) adsorbed anti-(Mem_H-Bel_N) IgG to HA_2 and HA_1. Serial dilutions of IgG were added to wells of a polyvinyl tray coated with Mem HA_1 (□——□), Mem HA_2 (○——○) or PBS (△——△). Normal rabbit IgG (○---○) was also added to wells coated with Mem HA_2. Bound IgG was detected by addition of radioiodinated protein A. (From Brown et al. J. Immunol. 1980 125 with permission).

a comparison of the lateral displacement of titration curves between HA_1 and HA_2 suggests that only a small proportion of the total antibody is directed against HA_2.

In order to determine which portion of the HA_2 chain carries antigenic activity a different experimental approach was employed. In an inhibition assay, HA_2 was demonstrated to prevent the binding of anti-viral antibody to fragment P1 (Fig. 3). As the only CNBr peptide from HA_2 present in P1 is

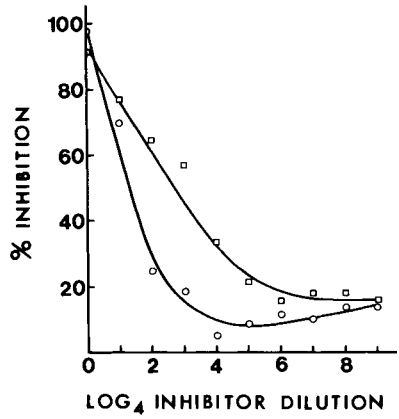

FIGURE 3. Effect of Mem HA_1 and HA_2 on the binding of shearwater adsorbed anti-(Mem_H-Bel_N) IgG to P1. Radiolabeled P1 (0.1 pmoles), together with sufficient IgG to bind 50% of the radiolabeled antigen, was incubated in the presence of dilutions of HA_1 (□—□) or HA_2 (○—○). Antigen-antibody complexes were detected by addition of protein A-Sepharose. (From Brown et al. J. Immunol. 1980 125 with permission).

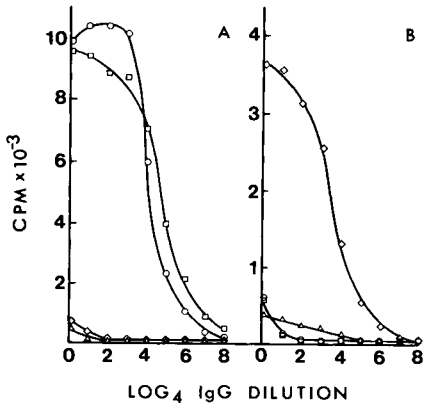

FIGURE 4. Specificity of antibody to HA_2. Dilutions of (A) shearwater-adsorbed anti-(Mem_H-Bel_N) IgG and (B) shearwater-adsorbed anti-(Jap_H-Bel_N) IgG were added to polyvinyl wells coated with Mem(H3) HA_2 (○—○), X-31(H3) HA_2 (□—□) Jap(H2) HA_2 (◇—◇) and Bel(H1) HA_2 (△—△). Bound IgG was detected by addition of radioiodinated protein A. (From Brown et al. J. Immunol. 1980, 125 with permission).

HA_2CN1 it is concluded that this region of the HA molecule, comprising the C-terminal 90 amino acids, is antigenically active. Recent data for HA_2 (8-13) shows that the amino acid sequences of members of a particular subtype are highly conserved but that there are considerable differences between subtypes. These findings are compatible with our results that antibodies directed against Mem(H3) react with HA_2 from X-31(H3) but not with HA_2 from Jap(H2) or Bel(H1) viruses. Also, antibodies directed against H2 react with HA_2 from this subtype only (Fig. 4). These results indicate that the determinant(s) on HA_2 is subtype-specific.

The Carbohydrate Antigenic Determinants

Attached to the polypeptide backbone of the HA glycoprotein of A/Mem/102/72 are seven separate oligosaccharide side-chains (9). Antibodies are elicited by these side-chains and cross-react both with the carbohydrate of chicken egg-grown influenza viral HA from different subtypes and with "host antigen" (7). The occurrence of carbohydrate as seven separate side-chains raises the question of whether each is antigenic or whether activity is restricted to one or a few side-chains. This issue is dealt with in detail elsewhere in this volume (Brown et al) but it is useful at this point to note that antigenic activity is restricted to only three of the seven side-chains at positions 8 and 22 in HA_1 (Fig. 5) and position 154 in HA_2

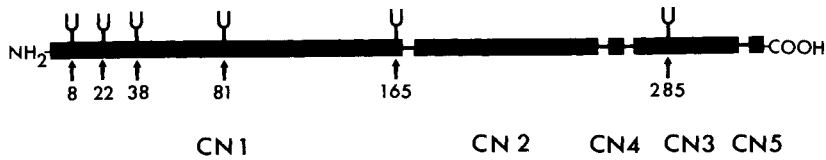

FIGURE 5. Schematic diagram showing location of the oligosaccharide side-chains within the CNBr-derived fragments of A/Memphis/102/72 HA_1.

and that these are all of the N-acetyllactosamine or "complex" type.

Reaction of HA and its Fragments with Monoclonal Antibodies

Because monoclonal antibodies are directed against a single epitope, the development of hybridoma technology has presented us with novel approaches to the delineation of protein antigenic determinants. A selection of monoclonal antibodies directed against X-31 provided by Dr. Walter

Gerhard were titrated for HI activity against X-31 and Mem_H-Bel_N viruses (Table 2). These monoclonal antibodies were also tested by radioimmunoassay for binding to isolated

Table 2. Characteristics of Monoclonal Antibodies

Monoclone	Code	Immunogen	HI Titre[a] against X-31	HI Titre[a] against Mem_H-Bel_N
H14 A11-5	2	X-31	80	905
H14 A13-15	3	X-31	51,200	1,600
H14 A20-14	4	X-31	512,000	1,131
H14 A21-3	5	X-31	362,000	256,000
H14 B1-10	6	X-31	10	10
H14 B10-1	7	X-31	20	905
H14 B17-16	8	X-31	18,100	400
H14 B18-12	9	X-31	36,200	1,131
H14 B23-13	10	X-31	20	905

[a]Reciprocal of the dilution of monoclonal antibody inhibiting four HA units of virus.

HA, HA_1, HA_2 and CNBr fragments of HA (Figs. 6 and 7).

Although each monoclonal antibody is capable of binding X-31 virus and its HA, only certain of them bind to HA_1 none to HA_2 and none (except monoclone 6) to pooled or individual CNBr-derived fragments. This result suggests that antigenic features previously recognized by some of the monoclones are lost following separation of HA_1 and HA_2 . This loss in binding does not appear to correlate with the cross-reactive or HI properties of the monoclonal antibodies.

In no case did HA_1 lack antigenic activty when tested against hyperimmune sera from rabbits or mice. In the hope that simultaneous binding of monoclonal antibodies to distinct epitopes might synergistically restore conformation and hence antigenic activity to the HA_1 chain, a number of monoclones, individually incapable of binding HA_1, were pooled and tested, but again no binding was detected.

Monoclone 6 (H14 B1-10) is unusual as it bound to the CNBr digest of HA as well as to HA_1, HA and virions of both X-31(H3) and Bel(H1). This strongly suggested that monoclone 6 is directed against the cross-reactive carbohydrate antigen. Additional proof for the anti-carbohydrate nature of this antibody is presented in the next section.

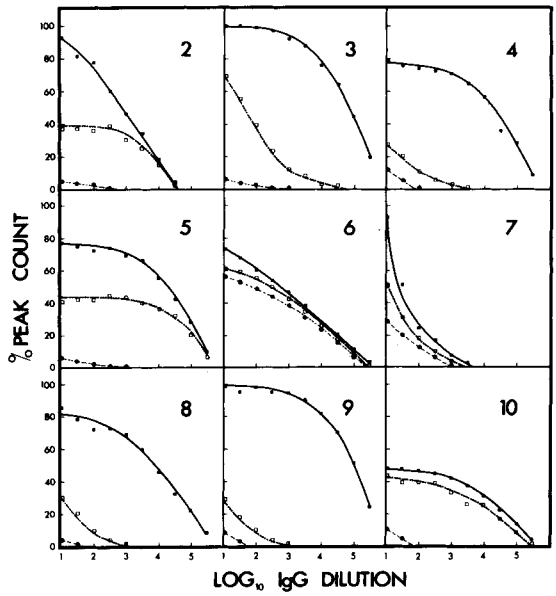

FIGURE 6. Binding of anti-(X-31 virus) monoclonal antibodies (2-10) to HA. Serial dilutions of individual monoclonal IgG preparations were added to polyvinyl wells coated with the HA of X-31(H3) (■——■), Mem/102/72(H3) (□·····□) or Bel/42(HO) (●------●). Bound antibody was detected by addition of radioiodinated rabbit anti-(mouse IgG) IgG.

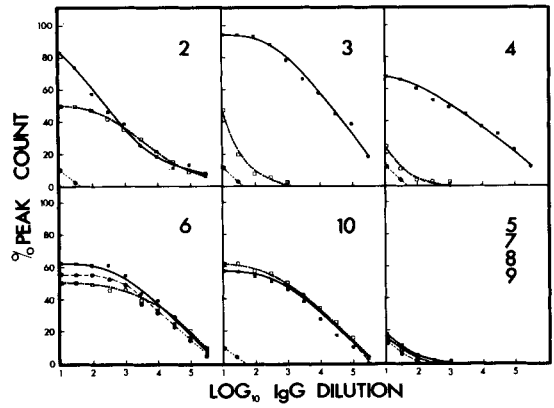

FIGURE 7. Binding of monoclonal antibodies (2-10) to HA_1. Symbols as in Fig. 6.

Enumeration of the Separate Antigenic Sites on HA

We have previously reported (14) a study utilising a competitive radioimmunoassay to demonstrate that "strain-specific" and "cross-reactive" antibodies obtained from hyperimmune sera may bind to the same antigenic site(s) on the HA molecule. The availability of a panel of monoclonal antibodies to HA now provides an opportunity for exploiting this particular assay to determine the number of discrete antigenic regions on HA and also perhaps to comment on their topography. The rationale of this approach is that if two monoclonal antibodies recognise the same determinant then an excess of one will inhibit the binding of the second. Such inhibition can be detected if the second antibody is radiolabeled. If on the other hand, the two antibodies recognise totally different determinants which are distant, then inhibition will not occur. An intermediate situation may also arise when two non-identical epitopes are sufficiently close such that binding of an IgG molecule to one will sterically hinder the attachment of antibody to the second epitope.

Pure IgG was prepared from each monoclonal antibody preparation, radioiodinated and titrated for its ability to bind to X-31 HA in the presence of dilutions of unlabeled antibody of a different clone (Fig. 8).

The results with this small number of monoclonal antibodies allows the clear delineation of four distinct antigenic sites on X-31 HA. The first site is defined by monoclones 2 and 10 which compete completely and reciprocally with one another but with none of the other monoclones tested (Fig. 8a). The second is the binding site for monoclonal antibodies 3,5,8 and 9 which also block one another completely and reciprocally (Fig. 8b) despite the fact that some are relatively specific for X-31 whereas others show cross-reaction with X-31 and Mem. (Fig. 6 and Table 2). A third site is indicated by the behaviour of monoclone 4 (H14 A20). This antibody is not inhibited by any of the other preparations (Fig. 8c) whereas monoclone 4 is able to block monoclones 3 and 9 (Fig. 8d). The fourth site is defined by monoclone 6 and is the carbohydrate determinant. The evidence for this is that monoclonal antibody 6 (i) binds to Bel(H1) as well as to X-31(H3) virus, HA, HA_1 and a CNBr digest of HA (Figs. 6 and 7), (ii) does not have significant HI activity (Table 2), and (iii) blocks and is blocked by IgG obtained from a rabbit immunized with purified carbohydrate "host antigen" from uninfected eggs.

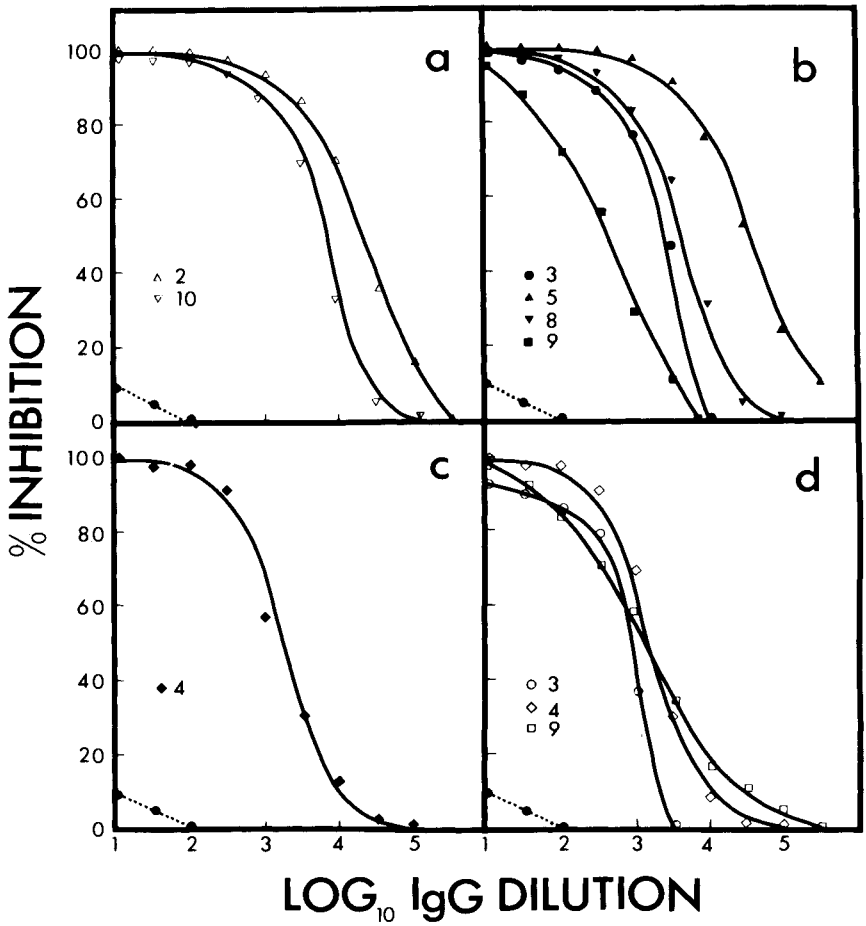

FIGURE 8. Radioiodinated IgG from hybridomas 2(a), 5(b), 4(c) or 3,4 and 9(d) was mixed with serial dilutions of unlabeled IgG from the hybridomas listed on each panel. The mixtures were added to polyvinyl wells coated with X-31 HA. The Figure shows the percentage inhibition of binding of labeled monoclone by each dilution of unlabeled monoclone. The broken line indicates the inhibition achieved by all other monoclones not individually cited.

Synergistic Binding of Monoclonal Antibodies

If, instead of using unlabeled hybridoma antibody in large excess to examine its capacity to block the binding of a second labeled monoclone, both monoclones are employed at marginal concentration, the phenomenon that certain monoclones actually enhance the binding of others is observed. Table 3 shows selected data from a typical single

Table 3. Synergistic Binding of Monoclonal Antibodies

	^{131}I-Monoclone					
^{125}I-Monoclone	*2*	*3*	*4*	*5*	*9*	10
2	*-75*	*+45*	*+49*	*+88*	*+97*	*-70*
	-45	-13	-44	-47	-36	-58
3	*-42*	*-40*	*+23*	*+55*	*+5*	*-30*
	+16	-42	-9	-41	+4	+32
5	*-38*	*-72*	*+80*	*-58*	*-76*	*-9*
	+45	+52	+80	-36	+83	+76
8	*-16*	*+29*	*+86*	*+112*	*+68*	*-11*
	+65	+40	+40	-35	+6	+60

Results are expressed as percentage enhancement (+) of binding or percentage inhibition (-) of binding relative to the level of binding obtained with a single antibody.

experiment in which 25 ng of IgG from each of two hybridomas labeled with ^{125}I and ^{131}I respectively were tested for simultaneous binding to X-31 HA.

It is seen that whereas ^{125}I and ^{131}I-labeled preparations of the same monoclone inhibit the binding of one another, pairs of different monoclones may either inhibit or enhance the other's binding; this is not always reciprocal.

Synthesis of an Antigenically Active Peptide

In view of the frequency with which amino acid substitutions occur in the "loop" region within HA_1CN1 (15,16), synthesis of a peptide corresponding to residues 140-150, with the sequence NH_2-LYS-ARG-GLY-PRO-GLY-SER-GLY-PHE-PHE-SER-ARG-COOH in X-31 was carried out by Dr. Geoffrey Tregear using the Merrifield solid phase method. The synthesised peptide was extremely soluble and readily polymerised using glutaraldehyde as a bifunctional

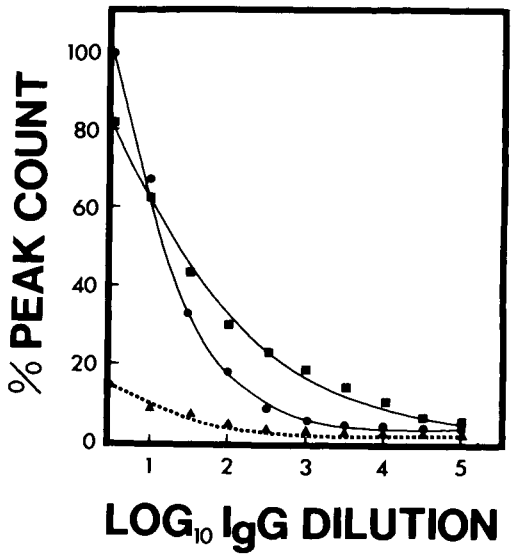

FIGURE 9. Binding of anti-(X-31 virus) IgG to a polymerised synthetic peptide, NH_2-LYS-ARG-GLY-PRO-GLY-SER-GLY-PHE-PHE-SER-ARG-COOH. Serial dilutions of IgG obtained from two rabbits (■——■) and (●——●) inoculated with X-31 virus and from a pre-immune animal (▲- - - - - - - -▲) were added to wells coated with glutaraldehyde cross-linked synthetic peptide. Bound IgG was detected by addition of radiolabeled protein A. Results are expressed as a percentage of the maximum count achieved.

reagent. The polymerised form of the synthetic peptide was used to coat wells of a polyvinyl plate to which was then added IgG from antisera of rabbits immunized with X-31 virus; binding was assayed by ^{125}I-protein A (Fig. 9).

The bond between the C-terminal arginine of the synthetic peptide and the resin to which it is attached during the solid phase synthesis procedure is somewhat resistant to the trifluoroacetic acid cleavage step, hence it proved possible to obtain the peptide coupled to resin in either blocked or deblocked form. When radiolabeled IgG obtained from rabbit anti-X-31 serum was passed through a sample of each of these resins it was found that antibody bound to the de-blocked resin to a much greater extent than to the blocked resin.

Inoculation of rabbits with synthetic peptide, glutaraldehyde-polymerised synthetic peptide, or synthetic peptide conjugated to various carriers has not yet elicited antibodies capable of binding to virus, nor have any of the available monoclonal antibodies yet bound to any form of the synthetic peptide. Nevertheless, the demonstration that antigenic activity is associated with the polymerised form of this short synthetic peptide is encouraging and additional peptides are currently being synthesised to explore the antigenic structure of influenza virus HA using this approach.

DISCUSSION

Each level of structural organisation within a protein provides the requirements for immunochemical specificity. In some cases, a particular sequence of amino acids is all that is required for recognition by antibody whereas in others a more definite structural feature such as alpha-helix or beta-sheet is required. The very subtle spatial arrangement of globular proteins provides a third level of organisation where certain regions of the polypeptide are brought into juxtaposition through the correct folding of the molecule; disruption of tertiary structure often results in complete loss of antigenic activity. Even more elusive perhaps are the alterations in antigenicity observed when conformational changes associated with molecular function occur; for example, the oxygenated and deoxygenated forms of hemaglobin are antigenically distinct. Furthermore, antibodies to one conformational form can induce it to assume a different conformation (17). Finally, oligomeric proteins may express a unique class of antigenic determinants not present in monomeric proteins. These include the interface surfaces involved in subunit-subunit interactions and also sites exposed as a result of conformational changes that the subunits experience during dissociation (18,19).

Each of these features exist in the hemagglutinin molecule (15,16) and are candidates for sites of antigenic activity.

Although there is not yet direct evidence which allows us to identify the sites on HA to which antibodies bind, it is interesting to note that although a cyanogen bromide digest of HA_1 is antigenically active, this activity is abrogated by cleavage of disulfide bonds. This would seem to suggest that there are few, if any, antigenic determinants within HA which are dependent only upon primary structure.

The overall tertiary structure of HA appears to be an important characteristic for antibody binding since the larger fragments such as HA_1 and P1 possess substantial activity. Both these species would be expected to retain a substantial portion of the conformational integrity of the complete molecule due to their size and the stabilizing effects on conformation exerted by disulfide bonds. As the method of preparation of HA_1CN1 undoubtedly results in loss of tertiary structure the noticeable decrease in activity of this fragment supports the concept that conformational determinants are central to the antigenicity of HA.

Although not evidence for the existence of conformational determinants or otherwise, it is at this point tempting to speculate that the enhancement of binding of one monoclonal antibody to HA in the presence of a second monoclonal antibody might reflect a rearrangement in the conformation of the antigen effected by one antibody which allows a second IgG molecule to bind more efficiently. Perhaps such a phenomenon could explain why binding of IgG from polyclonal sera to fragments of HA readily occurred whereas binding of monoclonal antibody was not detected.

An intriguing feature of the HA model (15,16) is the region consisting of two single anti-parallel peptide chains which serve to connect the globular head to the rest of the molecule. Possibly during hemagglutination or binding to cell surface receptors, the trimer undergoes a large scale hinge-bending motion at this region. It will be interesting to see if any evidence of antigenically distinguishable forms of HA can be detected when any conformational changes associated with the trimer's molecular functions occur. Similarly, any partial dissociation of the trimer which may be associated with molecular function may expose or destroy additional antigenic sites.

The competitive binding assay has allowed us to define three separate and spatially distinct antigenic sites occurring in the polypeptide of X-31 HA and to demonstrate that the carbohydrate antigens are separate from these regions. It remains to be seen whether use of a larger panel of monoclonal antibodies will allow us to characterise additional antigenic sites. Furthermore Fab fragments, which will offer less steric hindrance in competitive binding assays may reveal subtle spatial relationships between different epitopes within a given antigenic sites.

Perhaps the most definitive way of delineating those regions of the polypeptide to which antibody binds is to synthesise peptides and then examine their immunogenic and antigenic properties. In the case of the undecapeptide corresponding to the "loop" region of the HA molecule (15,16)

which we have synthesised, significant antigenic activity was detected using rabbit anti-viral IgG. No activity was detected, however, using mouse monoclonal antibodies, even monoclone 4 (H14 A20) which is known to facilitate the selection of variants which have an amino acid change at position 143 in HA_1 (20). In the light of the failure of monoclones to bind to much larger fragments of HA however this last result is not surprising. Experiments are currently underway to extend our repertoire of synthetic peptides with a view to defining more precisely the important antigenic sites on the influenza HA molecule.

ACKNOWLEDGEMENTS

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REFERENCES

1. Laver, W.G. Virology 45, 275 (1971).
2. Jackson, D.C., Russell, R.J., Ward, C.W. and Dopheide, T.A. Virology 89, 199 (1978).
3. Jackson, D.C., Dopheide, T.A., Russell, R.J., White, D.O. and Ward, C.W. Virology 93, 458 (1979).
4. Jackson, D.C. J. Imm. Meth. 34, 253 (1980).
5. Brown, L.E., Dopheide, T.A., Ward, C.W., White, D.O. and Jackson, D.C. J. Immunol. 125, 1583 (1980).
6. Jackson, D.C., Brown, L.E., White, D.O., Dopheide, T.A. and Ward, C.W. J. Immunol. 123, 2610 (1979).
7. Jackson, D.C., Brown, L.E., and White, D.O. J. gen. Virol. 52, 163 (1981).
8. Porter, A.G., Barber, C., Carey, N.H., Hallwell, R.A., Threlfall, G. and Emtage, J.S. Nature 282, 471 (1979).
9. Ward, C.W. and Dopheide, T.A. Virology 103, 37 (1980).
10. Dopheide, T.A. and Ward, C.W. in "Structure and Variation in Influenza Virus". (Laver and Air, eds.) p.21. Elsevier, North Holland (1980).

11. Min-Jou, W., Verhoeyen, M., Deuos, R., Saman, E., Huylebroeck, D., van Rompuy, L., Fang, R.X. and Fiers, W. in "Structure and Variation in Influenza Virus". (Laver and Air, eds.) p.63. Elsevier, North Holland (1980).
12. Gething, M-J., Bye, J., Skehel, J.J. and Waterfield, M.D. in "Structure and Variation in Influenza virus". (Laver and Air, eds.) p.1. Elsevier, North Holland (1980).
13. Sleigh, M.J., Both, G.W., Brownlee, G.G., Bender, V.J. and Moss, B.A. in "Structure and Variation in Influenza Virus". (Laver and Air, eds.) p.69. Elsevier, North Holland (1980).
14. Russell, R.J., Burns, W.H., White, D.O., Anders, E.M., Ward, C.W. and Jackson, D.C. J. Immunol. 123, 825 (1979).
15. Wiley, D.C., Wilson, I.A. and Skehel, J.J. Nature 289, 373, (1981).
16. Wilson, I.A., Skehel, J.J. and Wiley, D.C. Nature 289, 366, (1981).
17. Crumpton, M.J. in "The Antigens" (Sela, M.ed.) p1. Academic Press, New York, Vol.II (1974)
18. Bartel, A.H. and Campbell, D.H. Arch. Biochem. Biophys. 82, 232 (1959).
19. Hahn, E. and Timpl, R. Eur. J. Immunol. 3, 442 (1973)
20. Laver, W.G., Air, G.M., Webster, R.G. Gerhard, W., Ward, C.W. and Dopheide,T.A. in "Structure and Variation in Influenza Virus". p.295. Elsevier, North Holland (1980).

ANTIGENIC CHARACTERIZATION OF INFLUENZA A VIRUS NUCLEOPROTEIN[1]

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ABSTRACT

Antigenic variation in the nucleoprotein (NP) of influenza A viruses was studied using monoclonal antibodies. Enzyme-linked immunosorbent assays (ELISA) of these antibodies indicated that the WSN/33 (HON1) influenza virus NP possesses at least five distinct antigenic determinants, and that variation in some of these determinants has occurred in other influenza A strains. Competitive binding ELISA assays showed that the five determinants comprise three non-overlapping antigenic domains on the NP molecule. Monoclonal antibodies to two of the NP domains inhibited viral RNA synthesis *in vitro*, suggesting that these specific regions of the NP molecule are topographically or functionally involved in RNA transcription.

INTRODUCTION

The three types of influenza viruses (types A, B, and C) are distinguished by lack of serological cross-reactivity among the type-specific antigens of their internally located matrix proteins and nucleoprotein (NP). Initial complement fixation and immunodiffusion tests indicated that the NP and matrix proteins of influenza A viruses are antigenically stable (1,2). However, reports of differing electrophoretic migration rates of NP (3-5) and of RNA segments coding for NP (6) in polyacrylamide gels and reports of differences in NP peptide maps (7) and in oligonucleotide maps of NP genes (8) from different influenza A virus strains indicate that mutations occur in the genetic information for the NP. The first direct evidence that this genetic variation is reflected in antigenic variability in the NP was obtained using immunodiffusion tests of purified NP and heterogeneous antiserum (9).

We have used monoclonal antibodies to examine antigenic variation in the influenza A virus NP and to determine the distribution of antigenic sites on the NP molecule. Since ribonucleoprotein (RNP) complexes in both infected cells and mature virions have transcriptase activity (10), we have also examined the affect of anti-NP antibodies on *in vitro* influenza virus RNA transcription.

METHODS

Viruses

The growth and purification of influenza viruses followed established procedures (11). The virus stock used for transcriptase assays was banded in ribonuclease-free sucrose gradients.

Production and Characterization of Monoclonal Antibodies to the NP.

Hybridoma cell lines were produced by fusing splenic lymphocytes obtained from Balb/c mice immunized with A/WSN/33 (HON1) influenza virus with P3/X63-Ag8 cells using the methods of Kohler and Milstein (12). Cell lines secreting antibodies directed to the influenza virus NP were identified by radioimmunoprecipitation assays, as described previously (13). Indirect enzyme-linked immunosorbent assays (ELISA) of these antibodies with NP purified by polyacrylamide gel electrophoresis, and also with X37 and X47 recombinant influenza viruses (14) eliminated the possibility that any of the antibodies were directed to influenza virus P1, P2, or P3 proteins. Indirect ELISA assays indicated that five monoclonal cell lines secreted antibodies which were each directed toward a different epitope on the NP molecule (13). These cell lines were grown as ascites tumors in pristane-primed Balb/c mice. Purified monoclonal antibodies were obtained by chromatography of ascites fluid on *S. aureus* protein A-conjugated Sepharose 4B columns.

Competitive-binding ELISA Assays

Purified A/WSN/33 (HON1) influenza virus in lysis buffer [0.05 M Tris-HCl (pH 7.5), containing 0.5% Triton X-100 and 0.6 M KCl] was adsorbed to polystyrene multiwell dishes. Monoclonal antibodies to the influenza A virus NP were conjugated to horseradish peroxidase. Prior to competitive-binding assays, each conjugated antibody preparation was titrated in direct ELISA tests. This was done by incubating dilutions of conjugates in individual wells of assays plates for 30 minutes. After washing to remove unbound conjugated antibodies, substrate solution [0.05 M citrate buffer (pH 4.0) containing 0.008% hydrogen peroxide and 40 mM azino-di-3-ethyl-benzothiazoline-6-sulfuric acid] was added. Optical densities at 405 nm of the resulting

mixtures were read on a multichannel photometer. In this manner, the antibody concentration required to saturate all available NP binding sites was determined for each conjugated antibody preparation.

Competitive-binding ELISA assays were performed using mixtures containing a fixed concentration of conjugated monoclonal antibodies (determined as above) and increasing concentrations of unconjugated, protein A-purified monoclonal antibodies. Mixtures were added to wells of ELISA assayed plates and assays as above.

RNA Transcriptase-inhibition Assays

The antibody-mediated inhibition assay of influenza virus RNA transcription was a modification of the method of Plotch and Krug (15), and is described in the legend to figure 1.

RESULTS

Monoclonal Antibodies Directed Toward Different Antigenic Determinants

Hybridoma cell lines secreting antibodies directed to the A/WSN/33 (HON1) influenza virus NP were identified by radioimmunoprecipitation tests as described previously (13). To determine whether the monoclonal antibodies were directed toward the same or different antigenic determinants on the NP molecule, they were tested in ELISA (Table 1). Tests of the monoclonal antibodies with a panel of influenza A viruses showed that although all reacted with the homologous WSN strain, five antibody preparations reacted with a different subset of heterologous virus strains (Table 1). For example, antibody 3/1 reacted only with the PR/8/34, FLW/1/52, and equine strains, whereas antibody 7/3 reacted only with the FM/1/47, FLW/1/52, and equine strains. Thus, each of the five monoclonal antibodies recognized a different antigenic determinant on the influenza virus NP molecule.

Antigenic Variation in NP Determinants

The above-described results indicated that some influenza A viruses do not possess all five of the determinants detectable on the NP of the WSN/33 strain. To study

Table 1. Unique reactivity patterns of five anti-NP monoclonal antibodies with influenza A viruses, as determined by ELISA

Influenza virus strain	Reactivity of monoclonal antibody					
	3/1	5/1	7/3	150/4	469/6	Reactivity of negative control
WSN/33 (HON1)	0.26[b]	0.27	0.22	0.32	0.27	0.04
PR/8/34 (HON1)	0.15	0.16	0.03[c]	0.13	0.14	0.04
FM/1/47 (H1N1)	0.04[c]	0.27	0.19	0.05[c]	0.05[c]	0.04
FLW/1/52 (H1N1)	0.18	0.20	0.26	0.05[c]	0.05[c]	0.04
USSR/90/77 (H1N1)	0.01[c]	0.19	0.03[c]	0.03[c]	0.02	0.04
Equine/Miami/63 (Heq2 Neq2)	0.21	0.19	0.22	0.25	0.05[c]	0.03
B/Hong Kong/5/72	0.01[c]	0.04[c]	0.02[c]	0.02[c]	0.04[c]	0.02

[a] The negative control was supernatant from P3/X63-Ag8 cells containing no anti-influenza virus antibodies. The average optical density at 405 nm produced by a control culture (P3/X63-Ag8) supernatant was 0.035. In evaluating the results of the ELISA, therefore, a monoclonal antibody producing an optical density of 405 nm of 0.07 was considered to be reactive in our assay.

[b] Optical density at 405 nm, as obtained by ELISA.

[c] These optical density values of 0.07 (less than twice that of negative controls) indicate that the monoclonal antibody has no reactivity with the viral NP.

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antigenic variation in the influenza A virus NP, we tested a larger panel of viruses by ELISA against the monoclonal antibodies (13). The viruses examined included representative field strains of the major human subtypes, animal strains, and laboratory strains. Results of these studies indicated that antigenic variation had occurred in the NP determinants of HONl, HlNl, H2N2, and H3N2 viruses.

In contrast to the variability detected in the NP of the human influenza viruses, we found only two examples of antigenic variation among animal strains. Although only a limited number of animal strains were tested, all but the equine strains were identical to the WSN virus in five determinants. Based on their reactivity with the monoclonal antibodies, all of the strains tested fell into six NP epitope groups (Table 2). Since the only NP alterations detected were those affecting five antibody-binding sites, the antigenic similarities among the viruses may be restricted to a minor, but variable, portion of the NP and do not necessarily reflect the total homologies among the NP genes of strains within an NP group. Antigenic variation in the NP appears to proceed independently of major antigenic changes in the hemagglutinin and neuraminidase. The NP epitope groups do not correlate with any particular hemagglutinin or neuraminidase subtype; most NP epitope groups are composed of strains representing several subtypes.

In some instances, viruses of the same surface antigen subtype have NP epitopes which are closely related to viruses of other subtypes. For example, the NP of some recent H1N1 isolates (California/10/78 and Fukushima/103/78) are more similar to the Texas/1/77 (H3N2) strain than to other currently circulating H1N1 strains (USSR/90/77 and Brazil/11/78). That the California/10/78 (H1N1) field strain has arisen by genetic reassortment between cocirculating H1N1 and H3N2 viruses and has derived its NP gene from an H3N2 source has been demonstrated (16).

Determination of the Number of Nonoverlapping Antigenic Domains on the Influenza A Virus NP

Competitive-binding ELISA assays were performed to investigate the distribution of five distinct antigenic determinants on the influenza A virus nucleoprotein. These experiments were designed to test the ability of each of five monoclonal antibody preparations to sterically inhibit the binding of antibodies to four other determinants on the

Table 2. Combinations of NP epitopes found in influenza A viruses circulating between 1933 and 1978

Group	NP epitopes recognized in ELISA by monoclonal antibody					Group members	Subtype
	3/1	5/1	7/3	150/4	469/6		
I	+	+	+	+	+	WSN/33	HON1
						Swine/Iowa/30	Hsw1N1
						Turkey/Oregon/71	Hav1Nav2
						Duck/Germany/1225/74	H2Nav1
II	+	+	-	+	+	PR/8/34	HON1
III	+	+	+	+	-	Bellamy/42	HON1
						Equine/Prague/1/57	Heq1Neq1
						Equine/Miami/1/63	Heq2Neq2
						Hong Kong/1/68	H3N2
IV	+	+	+	-	-	FLW/1/52	
						Fukushima/103/78	H1N1
						California/10/78	
						Japan/305/57	H2N2
						England/878/69	H3N2
						England/178/70	
						Texas/1/77	
V	-	+	+	-	-	FM/1/47	H1N1
						Aichi/2/68	H3N2
VI	-	+	-	-	-	FW/1/50	
						USSR/90/77	H1N1
						Brazil/11/78	

+ Optical density at 405 nm threefold higher than the optical density at 405 nm of P3/X63-Ag8 culture supernatant in ELISA --, optical density at 405 nm less than twice the optical density at 405 nm of a P3/X63-Ag8 culture supernatant in ELISA.
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NP molecule. The results of these experiments are illustrated by competitive binding curves (Fig. 1).

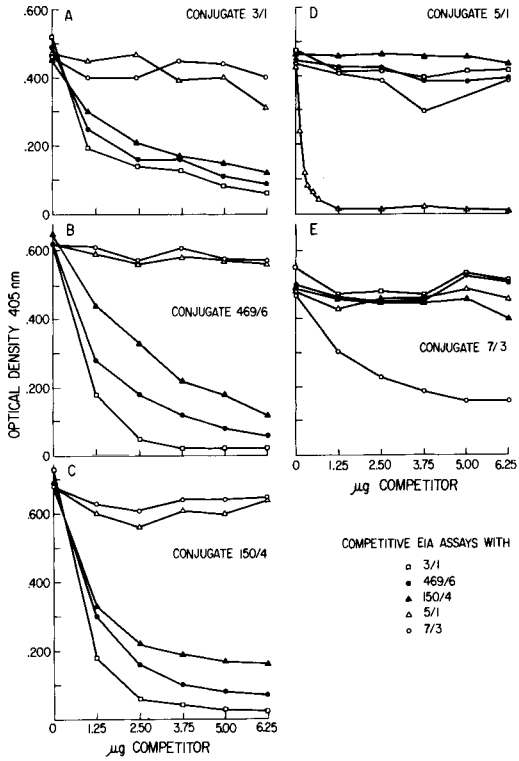

Figure 1. Determination of the number of nonoverlapping antigenic NP domains by competitive-binding ELISA. Fixed concentrations of conjugated antibodies were mixed with increasing concentrations of purified unconjugated antibody preparations. These mixtures were tested in direct ELISA with A/WSN/33 (HON1) virus as given in Methods.

The binding of two of the conjugated antibodies, 5/1 and 7/3 (Figs. 1D and E), was inhibited only by homologous antibody preparations, indicating that each recognizes an antigenic determinant which is physically distinct from the other four NP epitopes. In contrast, three monoclonal antibodies, 3/1, 469/6, and 150/4 (Figs. 1A, B, and C), were each capable of inhibiting the binding of the other

two. The results of these assays indicate that the influenza A virus NP possesses at least three separate antigenic regions. One of these regions is composed of three distinct epitopes, whereas each of the other two areas are currently defined by single antigenic determinants.

Inhibition of *in vitro* RNA Synthesis by Antibodies to the NP

The NP is an integral part of the transcriptase complex associated with influenza virions (10,17), but its function during RNA synthesis and its physical relationship with other proteins in the transcribing complex are unclear. To approach these questions, we examined the effect of monoclonal antibodies to the NP on *in vitro* RNA synthesis by A/WSN/33 (HON1) influenza virions. The results (Fig. 2) indicated that antibody 7/3 had no effect on the RNA polymerase reaction. In contrast, antibody 5/1 caused a 80% reduction in the amount of RNA synthesized. Antibodies 3/1, 150/4, and 469/6 also inhibited RNA transcription, although they did so less effectively than antibody 5/1. This inhibition was not due to ribonuclease contamination of the antibody preparations; inhibition of B/Hong Kong/77 influenza virus transcriptase was not observed (data not shown).

Kinetic studies (Fig. 3) demonstrated that addition of antibodies to virions prior to initiation of transcription or to transcribing virions at intervals after initiation produced comparable and immediate reduction in the rate of RNA synthesis. These results suggest that anti-NP antibodies inhibit elongation of nascent RNA, although the possibility also exists that they prevent initiation of RNA synthesis.

DISCUSSION

In contrast to the highly variable surface proteins, the influenza NP was characterized originally as antigenically invariant. Since the NP is located internally in the virion and antibodies to the NP do not neutralize virus infectivity, the apparent antigenic stability of this molecule seemed reasonable; it would not be exposed to immune selection pressure. Our results, however, clearly demonstrate that the influenza A virus NP possesses multiple antigenic determinants, four of which

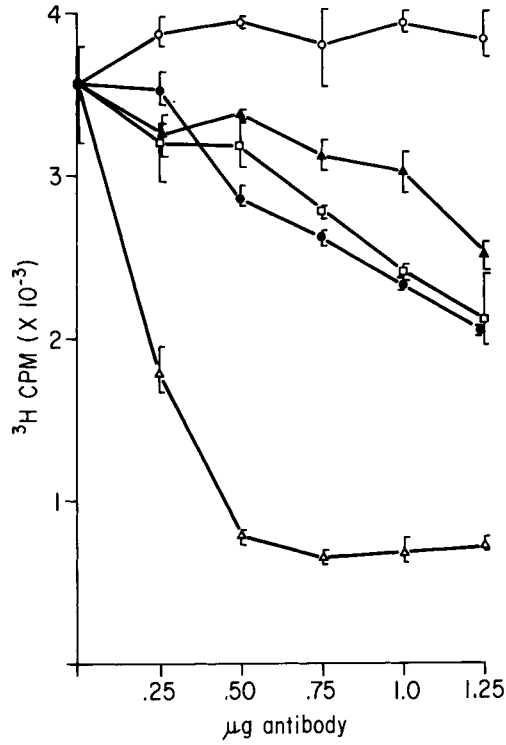

Figure 2. Inhibition of transcription by monoclonal antibodies to NP. Increasing concentrations of protein A-purified monoclonal antibodies (5 μl) were added to 40 μl of reaction mixture A [64 mM Tris-HCl (pH 8.0, 80 mM NaCl, 0.15% Triton X100, 0.8 mM dithiothreitol, 8.4 ug purified A/WSN/33 (HON1) influenza virus], and incubated at 4°C for 15 minutes. The transcriptase reaction was started by the addition of 5 μl of reaction mixture B [0.4 mM adenyl (3'-5') guanosine 0.8 mM $MgCl_2$, 0.64 mM adenosine, guanosine, and cytidine-triphosphate 0.8 mM uridine triphosphate and 2 μCi of ^{3}H-5'-uridine triphosphate. Reactions were stopped on ice after 15 minutes, and RNA was precipitated by the addition of trichloroacetic acid (TCA). Precipitates were washed three times with TCA and radioactivity was quantitated. Each point represents the average of three experiments; bars show the range of individual measurements (□ , antibody 3/1; ● , 469/6; ▲ ,150/4; △ , 5/1; and ○ , 7/3).

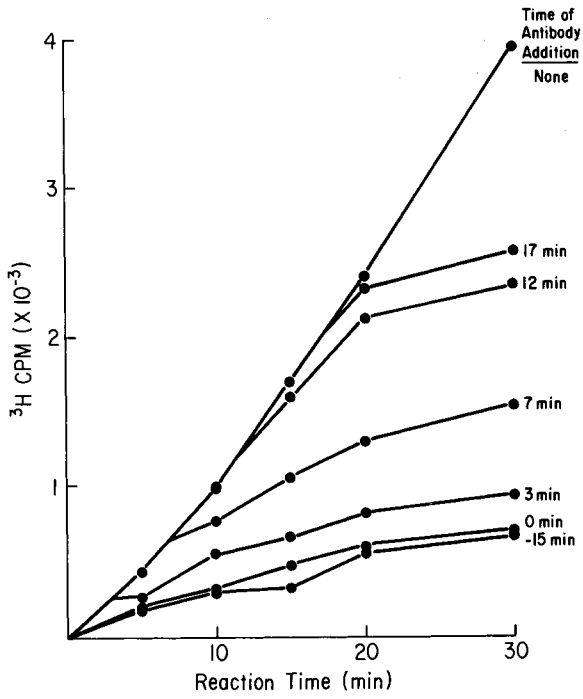

Figure 3. Kinetics of in vitro transcription by anti-NP monoclonal antibodies. Antibody 5/1 was added to individual transcriptase reaction mixtures at the indicated time intervals before or after initiation of viral RNA synthesis. ^{3}H-UTP incorporation into viral RNA was determined at the time of initiation, and also at 5, 10, 15, 10, and 30 minutes thereafter.

undergo variation in naturally arising human and animal field strains. A fifth determinant which has been detected on the NP of every influenza A virus examined appears to be an invariant type-specific determinant (13). Whether NP alterations offer influenza viruses a selective advantage in nature is not known. However, since the NP is intimately involved in the influenza virus replication complex and has been associated with virulence characteristics (18),

alterations in this molecule could affect the biological behavior of the virus.

Competitive antibody-binding assays indicate that the five detectable NP epitopes are distributed on the molecule in three non-overlapping antigenic domains. Antibodies to the three domains differ in their affects on in vitro RNA transcription.

The mechanism whereby anti-NP antibodies inhibit RNA synthesis is unknown. One possibility is that antibodies interfere directly with NP function. Although the precise function of NP is unclear, experiments using temperature-sensitive mutants (19-22) suggest that NP is involved in influenza virus replication. Furthermore, it is well documented that NP has a high binding affinity for influenza virus RNA (23) and is an integral component of functional transcriptase complexes (17). Since it appears likely that NP is an important cofactor in transcriptase activity, it is conceivable that the inhibition of RNA synthesis is due to direct inhibition of NP function. Alternatively, anti-NP antibodies could indirectly inhibit RNA synthesis by distortion of the helical NP-RNA structure or by prevention of the interaction of polymerase proteins with ribonucleoprotein complexes. On the other hand, these antibodies might sterically block enzymatic site(s) of polymerase enzymes in a manner analogous to the steric hindrance of influenza virus hemagglutination by anti-neuraminidase antibodies. Our results suggest that only antibodies directed to specific NP domains inhibit RNA synthesis. The data also suggest that the epitope corresponding to antibody 5/1 may represent an NP domain which is important for either structural or functional integrity of transcribing RNPs and may account for the apparent conservation of this epitope in influenza A viruses (13).

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REFERENCES

1. Schild, G.C., (1972) J. Gen. Virol. 15, 99.
2. Schild, G.C., and Pereia, H.G. (1969) J. Gen. Virol. 4, 355.

3. Almond, J.W., McGeoch, G.C., and Barry, R.D. (1977). Virology 81, 62.
4. Oxford, J.S., McGeoch, G.C., Schild, G.C., and Beare, A.S. (1978) Nature 273, 778.
5. Palese, P., Ritchey, M.B., and Schulman, J.L. (1977). Virology 76, 114.
6. Palese, P., and Schulman, J.L. (1976). J. Virol. 17, 876.
7. Dimmock, N.J, Carver, A.S., and Webster, R.G. (1980). Virology 103, 350.
8. Young, J.F., Desselberg, U., and Palese, P. (1979). Cell 18, 73.
9. Schild, G.C., Oxford, J.S., Newman, R.W. (1979). Virology 93, 569.
10. Bishop, D.H.L., Roy, P., Bean, W.J., and Simpson, R.W. (1971). J. Virol 10, 698.
11. Webster, R.G. (1970). Virology 42, 633.
12. Kohler, G., and Milstein, C., (1976). Eur. J. Immunol. 6, 511.
13. van Wyke, K.L., Hinshaw, V.S., Bean, W.J., and Webster, R.G. (1980). J. Virol. 35, 24.
14. Baez, M., Palese, P., and Kilbourne, E.D. (1980) J. Inf. Dis. 141, 362.
15. Plotch, S.J., and Krug, R.M. (1977). J. Virol. 21, 24.
16. Young, J.F., and Palese, P. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 6547.
17. Rochavansky, O.M. (1976). Virology 73, 327.
18. Bean, W.J., and Webster, R.G. in "Negative Strand Viruses and the Host Cell" (B.W.J. Mahy and R.D. Barry, eds.), p. 685. Academic Press, New York, (1978).
19. Krug, R.M. Ueda, M., and Palese, P. (1975). J. Virol. 16, 790.
20. Ritchey, M.B., and Palese, P. (1977). J. Virol. 21, 1196.
21. Sugiura, A., Tobita, K., Kilbourne, E.D. (1972). J. Virol. 10, 639.
22. Sugiura, A., Ueda, M., Tobita, K., and Enomoto, C. (1975). Virology 65, 363.
23. Scholtissek, C., and Becht, H. (1971). J. Gen. Virol. 10, 11.

GENES INVOLVED IN THE VIRULENCE OF AN AVIAN INFLUENZA VIRUS

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ABSTRACT

A/chicken/Japan/24 (H7N7) is a virus which was isolated from an outbreak of fowl plague-like illness among chickens and was subsequently found to have the virulence comparable to that of classical fowl plague virus. Intracerebral inoculation of up to 10 EID_{50} of chicken/Japan virus results in paralysis and death of chickens within 3 days. It also kills chick embryos within 48 hr of allantoic inoculation into embryonated eggs. Another avian virus, A/duck/Ukraine/1/63 (H3N8), in contrast, causes no illness, nor death in either chickens or embryonated eggs even with a large dose. In an attempt to segregate genes of chicken/Japan virus responsible for the observed virulence, we prepared recombinants between chicken/Japan and duck/Ukraine viruses. Parental derivation of genes in the recombinants was determined by urea-polyacrylamide gel electrophoresis of viral RNA. The virulence was tested by the following three criteria: lethality for chickens after intracerebral inoculation, lethality for embryos after allantoic inoculation into 15-day-old embryonated eggs, and lethality for embryos after allantoic inoculation into 10-day-old embryonated eggs. The recombinants in which HA, NA, and M genes had been derived from chicken/Japan virus and five other genes from duck/Ukraine virus were fully viru-

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lent by all three criteria. The recombinants which had received HA and NA genes from chicken/Japan virus showed a slightly but definitely diminished virulence toward chickens and 15-day-old embryos but the virulence for 10-day-old embryo was unchanged. The recombinants which had received only HA gene from chicken/Japan virus had a markedly diminished virulence for chickens and 15-day-old embryos. They were as lethal for 10-day-old embryos as chicken/Japan virus. It was concluded that HA gene from chicken/Japan virus was the key determinant of virulence, but in addition, NA and M genes were required for the full expression of virulence in 15-day-old embryos and chickens. Lethality for 15-day-old embryos closely paralleled the virulence for chickens, and was found to provide a useful and convenient marker of virulence.

INTRODUCTION

A large number of influenza viruses have been isolated from a variety of avian species (1, 2, 3, 4, 5). Most of them appeared to be quite harmless inhabitants of mainly intestinal tract of birds (6, 7, 8). A few viruses are, however, highly lethal. In recent years a number of influenza viruses were isolated also in Japan. These newly isolated strains were tested for pathogenicity by intracerebral injection of chickens (8). Virulent viruses were found only among those of the hemagglutinin (HA) subtypes of H7 (formerly Havl) and H5 (formerly Hav5). But not all members of these subtypes were virulent, in agreement with the previous finding (7). As these authors reported, the virulence appeared to be largely dependent upon the cleavability of the HA polypeptide, which enables the multicycle viral growth in a variety of tissues and organs. On the other hand, all human and animal influenza viruses so far tested were avirulent in chickens (8). It is worth noting that WSN virus is also avirulent. Because the neuraminidase (NA) of WSN facilitates the cleavage of the HA, this virus multiplies in many types of cell cultures, tissues and organs, including avian cells (9, 10, 11). Lack of pathogenicity of this virus in chickens shows that the cleavage of HA is not the exclusive determinant of virulence.

A/chicken/Japan/24 is the virus that was isolated from an epizootic of fowl plague-like illness among chickens. It causes an acute generalized infection resulting in paralysis and always death(8). Until recently it has been regarded as classical fowl plague virus itself. The present study is concerned with the question which gene(s) is responsible for the contrasting behavior of chicken/Japan from other avirulent avian influenza viruses.

METHODS

Virus strains Prototype virus strains were A/chicken /Japan/24 (H7N7) and A/duck/Ukraine/1/63 (H3N8) strains of influenza virus. Both viruses were cloned by plaque-to-plaque passage at 39.5°C in chick embryo (CE) cells. All subsequent propagation was at 37°C. Cloned virus stocks were prepared by allantoic infection of 10-day-old embryonated eggs.

Electrophoresis of virus RNA Virus RNA was labeled with ^{32}P during the viral growth in CE cells. To cultures infected with H3-containing viruses, 0.25 μg/ml of acetylated trypsin (Sigma Chemical Co.) was added. Virions were purified and viral RNA was extracted by the established method (12). Electrophoresis was done in 2.8% polyacrylamide gel containing 6M urea for 16 to 45 hr at room temperature (13). Either tris-phosphate-EDTA (14) or tris-borate-EDTA (15) buffer was used for gels and electrode buffer depending upon RNA segments to be resolved. Gels were dried and autoradiographed.

Virulence in chick embryos Serial 10-fold dilutions of virus to be tested were inoculated into the allantoic cavity of either 10-day- or 15-day-old embryonated eggs. Four eggs were used for each dilution. After 48-hr incubation at 37°C, embryos were taken out for inspection and LD_{50} was calculated.

Virulence for chickens Serial 10-fold dilutions of virus to be tested were inoculated intracerebrally into 10-day-old White Leghorn chickens. Four chickens were infected for each dilution. Chickens were housed in isolation chambers and kept for observation for 7 days. LD_{50} was calculated from the results of 4-day observation.

Table 1. Gene Derivation and Virulence of Recombinant Clones Obtained from the Cross between A/chicken/Japan/24 and A/duck/Ukraine/1/63.

	Gene derivation 1 2 3 4 5 6 7 8	EID_{50}/LD_{50}, Log_{10}		
		10-day-old embryos	15-day-old embryos	chickens
A/chicken/Japan /24 (H7N7)	J J J J J J J J	0	0.25	0
#27 (H7N7)	U J U J J J J J	0	0.25	0
#28	U U U J U J J J	0	0.50	0.25
#29	U U U J U J J U	0	0.50	0.50
A/duck/Ukraine /1/63 (H3N8)	U U U U U U U U	7.50	7.50	7.50

J: A/chicken/Japan

U: A/duck/Ukraine

RESULTS

Virulence of prototype virus strains Chicken/Japan virus causes a generalized infection in chickens that always ends fatally (8). Lethal infection follows either intracerebral or intramuscular inoculation. Since chickens developped illness sooner by the former route, the infection of chickens was done intracerebrally in this study. By this method, the virus of the dose as small as 10 EID_{50} was sufficient to kill chickens within 72 hr (Table 1). The virus also invaded and killed embryos in less than 48 hr when inoculated into the allantoic cavity of either 10-day- or 15-day-old embryonated eggs. In contrast, duck/Ukraine virus was avirulent for chickens by any route of infection. Nor did it invade embryos during allantoic infection. The two viruses behaved differently also in cell cultures. While chicken/Japan virus grew readily in CE cells, duck/Ukraine virus was incapable of multicycle growth unless trypsin was added to the culture medium. Polyacrylamide gel electrophoresis of virion polypeptides showed that the different growth patterns were caused by the different cleavability of the HA polypeptide of the two viruses. H7 polypeptide of chicken/Japan virus grown in CE cells was predominantly in cleaved forms, while H3 polypeptide of duck/Ukraine virus was not cleaved without the addition of trypsin.

All corresponding gene segments of the two viruses could be distinguished from each other by migration rate in urea-polyacrylamide gel electrophoresis, depending upon the electrophoretic buffer or the duration of electrophoretic run. The segment 4 of both virus RNAs coded for the HA, the segment 5 for the nucleoprotein (NP), the segment 6 for the NA, and the segments 7 and 8 for the matrix (M) and nonstructural (NS) proteins, respectively. Three largest segments corresponded to polymerase (P) proteins but further detailed assignment was not done.

Genetic cross between A/chicken/Japan/24 and A/duck/Ukrine/1/63 Recombinants of various genotypes were obtained between chicken/Japan and duck/Ukraine prototype strains. It was desired to obtain recombinants with maximal virulence, yet with minimal repre-

Table 2. Gene Derivation and Virulence of Recombinant Clones Obtained from the Backcross between #27 and A/duck/Ukraine/1/63.

	Gene derivation 1 2 3 4 5 6 7 8	EID_{50}/LD_{50}, Log_{10} 10-day-old embryos	15-day-old embryos	chickens
#31 (H7N7)	U U U J U J U U	0	1.50	1.50
#41 (H7N8)	U U U J U U U U	0	4.25	5.25
#46 (H7N8)	U U U J U U U U	0	4.75	6.25

sentation of chicken/Japan virus genome, in order to eliminate as many genes as possible that were not relevant to the virulence of this virus. Mixed infection of CE cells was done with UV-irradiated chicken/Japan and live duck/Ukraine viruses. The mixed yield was inoculated into the allantoic cavity of 15-day-old embryonated eggs. The preliminary study showed that the invasiveness and lethality of various avian influenza viruses to 15-day-old embryos on allantoic infection was correlated remarkably well with the virulence in chickens. Therefore, 15-day-old embryos was used as a convenient substitute for chickens. The virus was recovered from the brain of embryos. Plaques were isolated from CE cells kept under the agar overlay without trypsin.

Gene derivation and virulence of three representative recombinants are shown in Table 1. The virulence was assessed by the ratio of EID_{50} to LD_{50} in respective hosts. The three recombinant clones were all as virulent in 10-day-old embryos, 15-day-old embryos, and chickens as chicken/Japan virus, i.e. three P genes, NP and NS genes, were, therefore, not essential.

Backcross of #27 with A/duck/Ukraine/1/63 It was attempted to further segregate genes involved in the virulence by the second round of genetic cross. The mixed yield from the backcross of #27 with duck/Ukraine virus was plated on CE cells. Plaques which developped in the absence of trypsin were isolated. The results with three clones are shown in Table 2. When the M gene from chicken/Japan virus was replaced with that of duck/Ukraine virus leaving only HA and NA genes of the former (#31), the virulence in 15-day-old embryos and chickens was slightly diminished. When all genes were derived from duck/Ukraine virus except HA gene (#41 and 46), the virulence was drastically depressed. But the virulence in 10-day-old embryos remained unaltered. The HA gene from chicken/Japan virus alone is responsible for the virulence in 10-day-old embryos. To be virulent in 15-day-old embryos and chickens, however, NA and M genes from chicken/Japan virus was necessary.

Reconstitution of virulence In order to confirm the above conclusion, we attempted to restore the

Table 3. Gene Derivation and Virulence of Recombinant Clones Obtained from the Cross between #41 and #51.

	Gene derivation 1 2 3 4 5 6 7 8	EID_{50}/LD_{50}, Log_{10} 10-day-old embryos	15-day-old embryos	chickens
#51 (H3N7)	J J J U J J U J	7.50	7.50	7.50
#61 (H7N7)	U J J J J J U U	0	1.50	1.25
#62 (H7N7)	J J J J J J U J	0	1.75	1.25
#63 (H7N7)	U U U J J J U J	0	2.00	1.50

virulence by recombining the genes supposed to be involved. First, the recombinant virus with the genotype reciprocal to that of #41 was prepared. UV-duck/Ukraine virus was crossed with live chicken/Japan virus and the progeny virus was selected with anti-H7N8 serum. One clone, #51, nearly fullfilled the criteria. Its gene derivation was reciprocal to that of #41 except for M gene (Table 3). The complete lack of virulence of this recombinant by any standard confirmed the importance of the chicken/Japan HA. The cross between #41 and #51 was performed. The progeny clones capable of invading the brain of 15-day-old embryo were selected. Three such clones were obtained (Table 3). As expected, they have both HA and NA of chicken/Japan virus. Their virulence was increased beyond that of either parent, #41 and #51, and was comparable to that of #31. It was now confirmed that not only the HA but also the NA contribute to the virulence of chicken/Japan virus. The virulence of #61, 62, and 63 was still below that of chicken/Japan virus or virulent recombinants #27, 28, and 29, probably due to the lack of M gene from the virulent virus. This is particularly evident with #62 which had derived all genes from chicken/Japan virus except for M genes, yet manifested a reduced virulence in 15-day-old embryos and chickens.

DISCUSSION

Three genes, HA, NA, and M genes, were involved in the virulence of chicken/Japan virus as compared with duck/Ukraine virus. Among the three, the HA gene from chicken/Japan virus was the key determinant of virulence because of the unique susceptibility of HA of this virus to proteolytic cleavage in various tissues. In addition to the HA gene, NA and M genes were necessary as accessory factors for the full expression of virulence. However, the mechanism with which the NA and M genes are required remains to be determined.

The conclusion reached above differs in some respects from those of earlier studies (16, 17).

It could be due to the use of different virus strains. We have compared two virus strains both of avian origin. It was assumed that the comparison of two virus strains which were adapted to the same host species differing only in the virulence might be more appropriate for the study of virulence, because other factors like host range and the degree of adaptation had not to be considered.

Genetic studies utilizing recombinants are often confounded by different phenotypes within the same genotype, or clonal phenotypic variation. Such variation could arise from the heterogeneity of parent viruses. This is typically manifested as genetic dimorphism (18, 19). This source of ambiguity might be avoided by cloning of parent viruses before the genetic cross. Clonal phenotypic variation can also arise from mutational events during the genetic cross and the subsequent passages of progeny clones. To minimize ambiguity from such source, a selective screen might be used to discriminate against the virus with diminished virulence resulting from mutational events. We employed, as the selective screen, one passage of mixed yields in the brain of 15-day-old embryos. Furthermore, the involvement of genes in the virulence was confirmed by reconstitution of virulence. This experiment ruled out the fortuitously reduced virulence due to clonal phenotypic variation and established the causal relationship between the loss of either HA or NA gene and the reduced virulence. We wished to similarly prove the involvement of M gene. Unfortunately, however, an avirulent recombinant containing M gene from chicken/Japan virus has so far been unavailable.

REFERENCES

1. Slemons, R. D., Johnson, D, C,. Osborne, J. s., and Hayes, F. (1974). Avian Dis. 18, 119.
2. Easterday, B. C. (1975). In "Influenza Viruses and Influenza". (E. D. Kilbourne, ed.). pp. 449. Academic Press, New York.
3. Webster, R. G., Morita, M., Pridgen, C., and

Tumova, B. (1976). J. Gen. Virol. 32, 217.
4. Shortridge, K. F., Butterfield, W. K., Webster, R. G., and Campbell, C. H. (1977). Bull. W. H. O. 55, 15.
5. Hinshaw, V. S., Webster, R. G., and Turner, B. (1978). J. Gen. Virol. 41, 115.
6. Webster, R. G., Yakhno, M., Hinshaw, V. S., Bean, W. J., and Nurti, B. (1987). Virology 84, 268.
7. Bosch, F. X., Orlich, M., Klenk, H.-D., and Rott, R. (1979). Virology 95, 197.
8. Ogawa, T., Sugimura, T., Itohara, S., Tanaka, Y., and Kumagai, T. (1980). Arch. Virol. 64, 383.
9. Schulman, J. L., and Palese, P. (1977). J. Virol. 24, 170.
10. Sugiura, A., and Ueda, M. (1980). Virology 101, 440.
11. Nakajima, S., and Sugiura, A. (1980). Virology 101, 450.
12. Palese, P., and Schulamn, J. L. (1976). J. Virol. 17, 876.
13. Ueda, M., Tobita, K., Sugiura, A., and Enomoto C. (1978). J. Virol. 25, 685.
14. Floyd, R. W., Stone, M. P., and Joklik, W. K. (1974). Anal. Biochem. 59, 599.
15. Brownlee, G. G., and Cartwright, E. M. (1977). J. Mol. Biol. 114, 93.
16. Scholtissek, C., Rott, R., Orlich, M., Harms, E., and Rohde, W. (1977). Virology 81, 74.
17. Bean, W. J., and Webster, R. G. (1978). In "Negative Strand Viruses and the Host Cell", (B. W. J. Mahy, and R. D. Barry, eds.) pp. 685. Academic Press, New York.
18. Mowshowitz, S., and Kilbourne, E. D. (1975). In "Negative Strand Viruses", (B. W. J. Mahy, and R. D. Barry, eds.) pp. 765. Academic Press, New York.
19. Kilbourne, E. D. (1978). Proc. Natl. Acad. Sci. USA, 75, 6258.

SUPPRESSOR RECOMBINANTS AND SUPPRESSOR MUTANTS[1]

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ABSTRACT

Two temperature-sensitive (ts) mutants with lesions in segment 8 (NS gene) could be phenotypically suppressed by replacement of other segments derived from various influenza A strains. This phenomenon is called suppressor recombination. Furthermore, the reversion rate of various ts mutants belonging to different recombination-complementation groups was studied. For mutants carrying a ts lesion in segments 1 or 2 the reversion rate was extremely high, while for the mutant with a ts defect in segment 6 (neuraminidase (NA gene)) no significant reversion was found so far. Other mutants had intermediate rates. In several cases it could be demonstrated by backcross that reversion to wild type properties was due to suppressor mutation.

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INTRODUCTION

The genome of influenza A viruses consists of 8 single-stranded RNA segments of different molecular weights. Of the 7 larger segments each codes for one structural protein of the virus particle, while the smallest one carries the information for the synthesis of two nonstructural (NS) proteins (1,2, for a review see ref. 3). During double infection of an organism or cells in tissue culture by two different influenza A viruses the RNA segments can reassort freely (4), giving rise to the appearance of new influenza viruses (for a review see ref. 5). In rescue experiments with temperature sensitive (ts) mutants it was found that several pairs of RNA segments do not segregate easily, if the two parent viruses are only distantly related in the genes under investigation (6). This lack of segregation is best explained by cooperation of the corresponding gene products either in an enzyme complex composed of different polypeptides like the viral RNA polymerase, or during virus maturation (3). Indeed, if certain gene products cooperate well it should be possible to suppress a ts lesion phenotypically by a second mutation in that gene whose product cooperates. The first indication of such a suppressor mutant was found during study of a revertant isolated from a child vaccinated with a ts mutant (7). Furthermore, phenotypic suppression should also be possible by replacement of the total RNA segment whose gene product cooperates in some way with the ts product. Such an isolate should be called suppressor recombinant.

In this paper, we report on the isolation and characterization of such suppressor recombinants and mutants of influenza A virus.

METHODS

1. Virus Strains and Tissue Cultures. The following influenza A strains have been used: FPV (A/fowl plague/Rostock/34; Hav1N1); virus N (A/chick/Germany "N"/49; Hav2Neq1); DU (A/duck/Ukraine/1/63 ; Hav7Neq2); equi 2 (A/equine/Miami/1/68; Heq2Neq2); PR8 (A/PR/8/34; HON1).

The two ts mutants ts 451 and ts 526, which were used for the preparation of suppressor recombinants, were derived by mutagenesis from the possible partial heterozygote 19N. 19N has segments 3 and 6 derived from virus N as well as FPV, and segments 1, 2, 4, 7, and 8 only from FPV. There is evidence for a crossover in segment 5. 413 and 48 are homozygotes derived by plaque passages from 19N (10,11).

The following ts mutants of fowl plague virus or a recombinant thereof have been used: Lesion in segment 1 (Pol 1): ts 3, ts 115, ts 117, ts 132; lesion in segment 2 (Ptra): ts 90, ts 93; lesion in segment 3 (Pol 2): ts 263; lesion in segment 4 (hemagglutinin (HA) gene): ts 227, ts 1; lesion in segment 5 (nucleoprotein (NP) gene): ts 19, ts 81; lesion in segment 6 (NA gene): ts 113; lesion in segment 8 (NS gene): ts 412 (8,9):

Primary chick embryo cells (CEC), 48 hr after seeding, or MDCK cells were used.

2. Isolation of ts Mutants and Recombination Experiments. The procedure for the isolation of ts mutants by mutagenesis with 5-fluorouracil was essentially that published by Simpson and Hirst (12), as described in detail by Scholtissek et al. (13). Recombination frequencies were determined according to Sugiura et al. (14). Rescue experiments were performed by double infection of CEC at 33^{o} (p) or 40^{o} (np) with a ts mutant and a distinct prototype strain which does not form plaques on CEC. Plaque formers were further passaged at least three times before stocks in embryonated eggs were produced.

3. Determination of Gene Constellation of Recombinants. The gene constellation was determined by comparing the RNase protection of ^{32}P-labeled vRNA segments after hybridization with nonlabeled cRNA of the parent and recombinant strains (6,15).

RESULTS

1. Assignment of ts 451 and ts 526 to Recombination Groups

After mutagenesis of 19N with 5-fluorouracil two ts mutants (ts 451 and ts 526) were isolated, which were further characterized by double infection of CEC with a set of ts mutants with a known ts lesion. As shown in Table 1, ts 451 recombines (reassorts) with all ts mutants tested except ts 19 and ts 412. Ts 526 can be rescued with all ts mutants except ts 412. These results indicate that ts 451 has ts lesions at least in segments 5 (NP) and 8 (NS), while ts 526 has a ts lesion at least in segment 8. Since no standard ts mutant with a lesion in segment 7 is available, nothing can be said from these experiments concerning a ts lesion in segment 7.

TABLE 1. Recombination frequencies of ts 451 and ts 526 after double infection of CEC with a panel of standard ts mutants of FPV

Ts mutants	Recombination frequencies[a]							
	ts 3 segm. 1	ts 90 segm. 2	ts 263 segm. 3	ts 227 segm. 4	ts 19 segm. 5	ts 113 segm. 6	ts 412 segm. 8	Ts lesions in segment
ts 451	2	2	70	30	< 0.1	40	< 0.1	5 and 8
ts 526	10	6	4	30	50	50	< 0.1	8

[a]The recombination frequencies were calculated according to Sugiura et al. (14). Each value represents an average of 3 independent experiments.

2. Marker Rescue with Influenza Strains which do not form Plaques on CEC

In an attempt to confirm that the ts lesions in ts 451 are in segments 5 and 8, and in ts 526 in segment 8 only, we sought to prepare recombinants between these ts mutants and three other influenza strains which do not produce plaques on chick embryo cells. For this purpose ts^+ plaques were selected and cloned at 40° from cells doubly infected with ts mutants and PR8 (P) either at 33° (permissive = p) or at 40° C (nonpermissive = np). Further ts^+ recombinants were selected after double infection with equi 2 or DU at 40° C (= E or D, respectively). The derivation of the various RNA segments of these ts^+ isolates was determined by the hybridization technique (6). The results are listed in Table 2. The parent ts 526 has segment 3 derived from virus N and segments 1, 2, 4, 6, 7 and 8 from FPV. The parent ts 451 has also segment 3 derived from FPV. In both ts mutants segment 5 has about 65% of its sequence from virus N, and about 35% from FPV (10,11). Four of the 451 pP isolates (3,12,13, and 22) have all segments identical to the parent ts 451. At first sight they look like normal revertants. Only 451 pP24 has three segments derived from PR8, including segments 5 and 8. All of the 451 npP isolates have replaced at least segment 2 and 5, but only two of them also segment 8. All the recombinants between ts 526 and PR8 have replaced at least segment 2, but none of them segment 8. These results, at first sight, seem to contradict the recombination data summarized in Table 1: According to marker rescue with PR8 the ts lesion of ts 526 should reside in segment 2, not in segment 8 (Table 2); and in ts 451 the two ts lesions should reside in segments 2 and 5, not in 5 and 8 (Table 2). (It has been confirmed carefully that plaque formation after double infection with ts 90 at 40° is not due to intracistronic complementation (16): The plaques have the same morphology as 19N and they can be passaged at 40° indefinitely). The data on marker rescue of ts 526 with the two other influenza strains (DU and equi 2) are even more puzzling. They indicate that marker rescue with ts 526 does not give a clear answer concerning the assignment of a ts lesion to a particular vRNA segment.

TABLE 2. Derivation of the various genes after marker rescue of ts 451 and ts 526 with different influenza A strains[a]

Strain	vRNA segments							
	1	2	3	4	5	6	7	8
ts 451	F	F	F	F	N/F[b]	F	F	F
451 pP3	F	F	F	F	N/F	F	F	F
451 pP12	F	F	F	F	N/F	F	F	F
451 pP13	F	F	F	F	N/F	F	F	F
451 pP22	F	F	F	F	N/F	F	F	F
451 pP24	F	P	F	F	P	F	F	P
451 npP1	F	P	P	F	P	F	F	P
451 npP2	F	P	F	F	P	F	F	P
451 npP3	F	P	P	F	P	P	F	F
451 npP4	P	P	P	P	P	F	P	F
451 npP6	F	P	P	F	P	P	F	F
ts 526	F	F	N	F	N/F	F	F	F
526 pP6	F	P	N	F	N/F	P	F	F
526 pP7	F	P	P	F	N/F	F	F	F
526 pP10	F	P	N	F	N/F	F	F	F
526 pP23	F	P	N	F	N/F	F	F	F
526 pP26	F	P	N	F	N/F	F	F	F
526 npP2	F	P	P	F	N/F	F	F	F
526 npP3	F	P	P	F	N/F	F	F	F
526 npP4	F	P	P	F	N/F	F	F	F
526 npP5	F	P	P[c]	F	N/F	F	F	F
526 D1	D	D	D	F	N/F	F	F	F
526 D2	D	D	D[c]	F	N/F	F	F	F
526 D3	F	F	N[c]	F	N/F	F	F	D
526 D4	D	F	D[c]	F	N/F	F	F	D
526 D5	D	F	n.d.[d]	F	D	F	F	F
526 E2	E	F	E	F	N/F	F	F	F
526 E3	E	F	E	F	N/F	F	F	E

[a]CEC were doubly infected with ts mutants and PR8 (P) either at the permissive (p) or nonpermissive (np) temperature, or with DU (D) or equi 2 (E) at the nonpermissive temperature. The derivation of the RNA segments was determined by hybridization of ^{32}P-labeled vRNA segments of FPV or PR8 with an excess of nonlabeled cRNA of the recombinants etc. (6).

[b]N/F means crossover segment containing about 65% of the sequence derived from virus N and about 35% of the sequence derived from FPV (10).

[c]Derivation of segment 3 of the D recombinants is not completely certain, because of the very similar base sequence homology of virus N and DU to FPV (only 10% difference). [d]not done.

3. Backcross Analysis of Recombinants

The apparent discrepancy between the results of marker rescue and recombination might be solved, if one assumes that the gene carrying the ts defect is still present in the recombinants (segment 8) but that, by placing this defective segment into a new gene constellation, its phenotype might change to ts^+. Thus, the introduction of segment 2 of PR8 might enable its gene product to cooperate with the defective gene product(s) of the FPV-NS gene at 40°, while the segment 2 gene product of FPV can do so at 33°, but not at 40°. (With equi 2 the gene products of segments 1 and 3 might, correspondingly, cooperate with the defective NS-protein of FPV). If this hypothesis is correct then a backcross of such recombinants with the original parent 19N should yield a certain percentage of the progeny carrying the ts phenotype, namely those isolates in which segment 2 of PR8 has been replaced by the corresponding FPV segment. Therefore CEC were doubly infected with a recombinant and 19N (or a corresponding derivative thereof) at 33°. Fourteen hr later a plaque test was performed, using the supernatant medium and starting at 33° C. After 3 days at 33° C, the size of the plaques was marked on the bottom of the dish and the cultures were incubated for two further days at 40°. Thereafter the cultures were stained with neutral red, and the percentage of plaques which did not further grow at 40° was determined. Between 100 and 200 plaques were counted in each experiment, which was repeated at least once. Singly infected cultures were treated in the same way and are taken as controls. The results are listed in Table 3. In the supernatant of singly infected cells only about 1% of the plaques exhibit the ts phenotype. The same was found with cells doubly infected with the parent 19N and the recombinants carrying segment 8 of PR8. However, if recombinants with an unexchanged segment 8 were investigated, about 10% of the plaques did not grow further after the shift to 40° C. This indicates that in those recombinants the gene carrying the ts defect is still present and that the ts defect is located in segment 8.

Four plaques isolated from cells doubly infected with 526 pP6 and 19N, which did not grow further at 40° C, were picked and, without further purification, injected into embryonated eggs. When the allantoic fluids were analyzed three of them contained exclusively ts virus, while the yield of the fourth one still could multiply at 40° C. One of the ts isolates was further analyzed by recombination with the standard ts mutants as outlined in Table 1. Ts 412 was the only ts mutant which could not rescue the new isolate indicating that it carries the ts lesion solely

TABLE 3. Backcross of recombinants obtained after marker rescue of ts 451 and ts 526 with PR8

CEC infected with	Plaques growing at 40° C / plaques not growing at 40° C
19/N	250/3
431	193/1
451 pP3	84/1
451 pP3 x 19/N	107/9
451 pP12	82/0
451 pP12 x 19/N	121/11
451 pP13	136/2
451 pP13 x 19/N	130/20
451 pP22	198/1
451 pP22 x 19/N	254/15
451 pP24	a
451 pP24 x 48	203/2
451 npP1	a
451 npP1 x 19/N	113/2
451 npP3	155/2
451 npP3 x 19/N	114/5
451 npP6	172/2
451 npP6 x 48	118/15
526 pP6	159/2
526 pP6 x 19/N	83/15
526 pP7	192/2
526 pP7 x 19/N	169/18
526 pP10	115/1
526 pP10 x 19/N	116/13
526 pP23	186/1
526 pP23 x 431	82/13
526 pP26	162/2
526 pP26 x 431	132/17
526 npP2	165/1
526 npP2 x 19/N	165/14
526 npP5	209/2
526 npP5 x 19/N	180/14

[a]Plaques were not visible at 33° and therefore could not be marked. After staining about 200 plaques could be counted.

in segment 8. The gene constellation has been determined to be exactly the same as the parent ts mutant 526.

4. Multiplication of Recombinants in MDCK Cells

Ts 526 was isolated as a ts mutant on CEC. Like the parent 19N, it did not form plaques on MDCK cells at either 33° or 40° C (Table 4). When a plaque test with 526 pP7 was performed on MDCK cells, this recombinant turned out to be ts. At 40° C the plaque yield was about 10^{-5} that at 33°. With 526 pP6 or 526 pP10, the plaque yield at 40° was reduced maximally by a factor of 2 when compared with the yield at 33° C. 451 pP3, like the parent strain 19N, did not form plaques on MDCK cells at either temperature. The recombinant 451 npP6 again behaved like 526 pP6, being not ts on MDCK cells. All five recombinants which were ts^+ in CEC have different gene constellations (see Table 2). These results indicate that the host plays an important role in determining expression of the ts character of a virus.

5. Suppressor Mutation

When mutants with a ts lesion in different genes were studied with respect to the appearance of revertants, the reversion rate varied considerably (Table 5). The plaque morphology of many of the revertants was different from that of the wild type FPV. Especially with ts 3 almost all of the revertants formed turbid and fuzzy plaques at 40° C. In the case of the other ts mutants the revertants had sometimes a reduced plaque size, but the plaques were always clear with sharp borders. In seven allantoic fluids of eggs infected with ts 113 which were examined, no revertants were found. In preliminary experiments some of the revertants were studied by backcross. The results are listed in Table 6. Three of 5 revertants behaved like suppressor mutants in that the backcross again yielded nearly 10% ts plaques. These ts isolates have not yet been examined in detail. However, several of them exhibit a plaque morphology which is different from the original ts mutant. One isolate included in Table 6 was obtained during rescue of ts 19 with the Singapore strain (H2N2). This isolate contained all RNA segments of FPV. It formerly had been regarded as a chance revertant generated during rescue and plaque purification. It also behaved like a suppressor mutant. In the backcross

TABLE 4. Plaque tests of recombinants on CEC and MDCK cells[a]

Recombinant	CEC				MDCK			
	33°		40°		33°		40°	
	PFU	Ø[b]	PFU	Ø	PFU	Ø	PFU	Ø
19N	1×10^8	2	4×10^7	5	$< 10^3$	-	$< 10^3$	-
ts 526	2×10^8	2	$< 10^3$	-	$< 10^3$	-	$< 10^3$	-
ts 451	4×10^7	1	$< 10^3$	-	$< 10^3$	-	$< 10^3$	-
526 pP6	2×10^7	2	8×10^6	4	5×10^6	2	2×10^6	4
526 pP7	3×10^8	2	2×10^8	4	2×10^7	1	$< 10^3$	-
526 pP10	4×10^8	2	3×10^8	4	2×10^7	1	2×10^6	3
526 npP5	1×10^8	2	3×10^7	4	8×10^6	1	$< 10^3$	-
451 pP3	3×10^7	2	2×10^7	4	$< 10^3$	-	$< 10^3$	-
451 npP6	1×10^8	2	7×10^7	2	5×10^7	2	1×10^7	2

[a]The tissue cultures were stained 3 days after infection

[b]Approximate diameters of plaques in mm.

TABLE 5. Reversion rate of mutants with ts lesions in different genes[a]

ts mutant	lesion in segment	PFU $40^{o}/33^{o}$	ts mutant	lesion in segment	PFU $40^{o}/33^{o}$
ts 3 I	1	5×10^{-5}	ts 227 I	4	$<10^{-6}$
ts 3 II	1	4×10^{-4}	ts 227 II	4	$<10^{-6}$
ts 3 III	1	1×10^{-4}	ts 227 III	4	$<10^{-6}$
ts 3 IV	1	2×10^{-4}	ts 227 IV	4	$<10^{-6}$
ts 3 V	1	3×10^{-4}	ts 227 V	4	1×10^{-5}
ts 3 VI	1	1×10^{-5}	ts 227 VI	4	$<10^{-6}$
ts 3 VII	1	2×10^{-6}	ts 227 VII	4	$<10^{-6}$
ts 115 I	1	1×10^{-5}	ts 1 I	4	$<10^{-6}$
ts 115 II	1	1×10^{-6}	ts 1 II	4	$<10^{-6}$
ts 115 III	1	2×10^{-5}	ts 1 III	4	$<10^{-6}$
ts 117 I	1	1×10^{-5}	ts 1 IV	4	$<10^{-6}$
ts 117 II	1	5×10^{-6}	ts 1 V	4	1×10^{-5}
ts 132 I	1	2×10^{-6}			
ts 132 II	1	5×10^{-5}	ts 81 I	5	$<10^{-6}$
			ts 81 II	5	$<10^{-6}$
ts 90 I	2	1×10^{-4}	ts 81 III	5	1×10^{-5}
ts 90 II	2	1×10^{-4}	ts 81 IV	5	5×10^{-4}
ts 90 III	2	2×10^{-4}	ts 81 V	5	$<10^{-6}$
ts 90 IV	2	1×10^{-4}	ts 81 VI	5	1×10^{-5}
ts 90 V	2	1×10^{-5}	ts 81 VII	5	$<10^{-6}$
ts 93 I	2	1×10^{-4}	ts 81 VIII	5	4×10^{-5}
ts 93 II	2	1×10^{-4}	ts 19 I	5	1×10^{-6}
ts 93 III	2	5×10^{-5}	ts 19 II	5	$<10^{-6}$
			ts 19 III	5	$<10^{-6}$
ts 263 I	3	8×10^{-6}	ts 19 IV	5	2×10^{-6}
ts 263 II	3	8×10^{-6}	ts 19 V	5	$<10^{-6}$
ts 263 III	3	$<10^{-6}$	ts 19 VI	5	$<10^{-6}$
ts 263 IV	3	$<10^{-6}$			
ts 263 V	3	$<10^{-6}$	ts 412 I	8	$<10^{-6}$
ts 263 VI	3	2×10^{-6}	ts 412 II	8	$<10^{-6}$
ts 263 VII	3	2×10^{-6}	ts 412 III	8	$<10^{-6}$
ts 263 VIII	3	2×10^{-6}			
			ts 451 I	5,8	1×10^{-4}
			ts 451 II	5,8	1×10^{-5}
			ts 451 III	5,8	6×10^{-5}
			ts 451 IV	5,8	1×10^{-5}
			ts 451 V	5,8	$<10^{-6}$
			ts 451 VI	5,8	$<10^{-6}$

[a] Individual plaques of ts mutants were injected into embryonated eggs. Plaque tests were performed with these allantoic fluids, which had 10^{7}–10^{8} PFU/ml at 33°; $<10^{-6}$ means that no revertants have been recovered.

TABLE 6. Backcross of revertants of ts mutants[a]

CEC infected with	Plaques growing at 40° / Plaques not growing at 40°
3 R4	140/2
3 R4 x FPV	90/8
90 R1	138/2
90 R1 x FPV	169/10
90 R2	214/3
90 R2 x FPV	295/2
81 R1	308/3
81 R1 x FPV	285/4
81 R2	208/2
81 R2 x FPV	187/11
19 A2[b]	140/1
19 A2 x FPV	234/7

[a] For experimental details see Table 3. Five individual revertants (R) were investigated

[b] 19 A2 is an isolate with all 8 RNA segments of FPV, which was obtained during rescue of ts 19 with the Singapore strain.

with those isolates obtained during rescue of ts 451 with PR8 (pP3, pP12, pP13 and pP22) containing all segments of FPV (except for segment 5 which is N/F), about 10% ts mutants segregated (Table 3). Therefore these isolates, too, have to be regarded as suppressor mutants. Since the reversion rate of ts 451 is quite high (Table 5) considering that it is a double mutant, it might be expected that both mutations are suppressed at once. The ts isolates obtained after the backcross have not yet been examined.

DISCUSSION

Genes of influenza A viruses with a ts lesion have been transferred by reassortment to other strains in an effort to produce a live vaccine (17,18). However, it has been shown that reassortment may produce recombinants which have gained new properties which were not found in either of the parent strains, e.g. concerning pathogenicity or host range (for reviews see ref. 3 and 19, 20-23). Therefore, it might be expected that under certain circumstances a gene with a ts defect might lose its ts phenotype when transferred to another strain. Such a suppression of the ts phenotype has been observed with two independently isolated mutants of a FPV recombinant with a ts defect in the gene coding for the two NS proteins. After marker rescue of these two mutants with other influenza A strains, recombinants were isolated which have exchanged either segment 2 (PR8) or 1 and 3 (DU and equi 2), but not segment 8 which carries the ts defect as demonstrated by backcrosses with the parent strain. Thus the introduction of segment 2 or 1 and 3, respectively, of another influenza strain suppresses the ts phenotype located in segment 8. This might indicate that the polymerase complex and the NS proteins cooperate in a way which is not yet understood. These results furthermore demonstrate that by marker rescue the RNA segments cannot be assigned unequivocally to ts defects or corresponding virus proteins without proper backcrosses of the recombinants with the parent strains. Therefore, we have reexamined our recent assignments of ts defects to RNA segments (6) by backcrosses of corresponding recombinants with FPV. The original assignments have been reconfirmed in this way (Scholtissek, unpublished).

Besides suppressor recombination evidence for suppressor mutation was obtained which is in agreement with observations of Murphy et al. (7). The reversion rates of ts mutants with lesions in the genes coding for the polymerase complex (segments 1, 2, 3, and 5) are relatively high (see Table 5),

while those of the glycoproteins are rather low. It would be of interest to know how frequently reversion in the polymerase genes is due to suppressor mutation and thus to demonstrate cooperation of these gene products. A more extended study on this problem is underway. Here it is shown only that suppressor mutation can occur.

During our efforts to rescue ts 451 with PR8 at the permissive temperature (p), 4 out of 5 isolates turned out to be suppressor mutants (pP3, pP12, pP13, and pP22) which had not replaced any of their genes by PR8. Obviously this kind of suppressor mutation with ts 451 is not a rare event. Therefore we might assume that suppression was effective by a single point mutation affecting the expression of both ts lesions at once. During rescue at the nonpermissive temperature (npP) no such pseudorevertants could be isolated. The formation of pseudorevertants might be facilitated by the tendency to form partial heterozygotes which is a property of the parent strain 19N (10). A further indication for heterozygote formation is the observation that 451 npP4, after three cycles of plaque purification on CEC without trypsin lost the capacity to form plaques without trypsin after two further passages through embryonated eggs. This suggests that during the original plaque passages the isolates consisted of partial heterozygotes carrying at least segment 4 (HA gene) of both parents. During further passage in eggs the HA gene of FPV, which is required for plaque formation in CEC (24), must have been lost.

A further interesting aspect of these studies is that the suppression of the ts phenotype by recombination is dependent also on the host. Thus in CEC, but not in MDCK cells, infected with 526 pP7 the ts phenotype is suppressed. This indicates that a host factor might also be involved in this phenomenon.

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REFERENCES

1. Inglis, S. C., Barret, T., Brown, C. M., and Almond, J. W., Proc. Nat. Acad. Sci. USA 76, 3790 (1979).
2. Lamb, R. A., and Choppin, P. W., Proc. Nat. Acad. Sci. USA, 76 4908 (1979).
3. Scholtissek, C., Adv. Genetics 20, 1 (1979).
4. Nakajima, K., and Sugiura, A., Virology 81, 486 (1977).
5. Webster, R. G., and Laver, W. G., in "The Influenza Viruses and Influenza" (E. D. Kilbourne, ed.), p. 269. Academic Press, New York, (1975).
6. Scholtissek, C., Harms, E., Rohde, W., Orlich, M., and Rott, R., Virology 74, 332 (1976.
7. Murphy, B. R., Tolpin, M. D., Massicot, J. G., Kim, H.Y., Parrott, R. H., and Chanock, R. M., in "Genetic Variation of Viruses " (P. Palese and B. Roizman, eds)., Ann. New York Acad. Sci. 354, 172 (1980).
8. Scholtissek, C., and Bowles, A. L., Virology 67, 576 (1975).
9. Koennecke, I., Boschek, C. B., and Scholtissek, C., Virology 109, in press.
10. Scholtissek, C., Rohde, W., Harms, E., Rott, R., Orlich, M., and Boschek, C. B., Virology 89, 506 (1978).
11. Rohde, W., and Scholtissek, C., Arch. Virol. 64, 213 (1980).
12. Simpson, R. W., and Hirst, G. K., Virology 35, 41 (1968).
13. Scholtissek, C., Kruczinna, R., Rott, R., and Klenk, H.-D., Virology 58, 317 (1974).
14. Sugiura, A., Tobita, K., and Kilbourne, E. D., J. Virol. 10, 639 (1972).
15. Scholtissek, C., Rohde, W., von Hoyningen, V., and Rott, R., Virology 87, 13 (1978).
16. Heller, E., and Scholtissek, C., J. Gen. Virol. 49, 133 (1980).
17. Murphy, B. R., Wood, F. T., Massicot, J. G., and Chanock, R. M., Virology 88, 244 (1978).
18. Murphy, B. R., Wood, F. T., Massicot, J. G., and Chanock, R. M., Arch. Virol. 65, 175 (1980).
19. Kilbourne, E. D., Progr. Med. Virol. 5, 79 (1963).
20. Scholtissek, C., Vallbracht, A., Flehmig, B., and Rott, R., Virology 95, 492 (1979).
21. Rott, R., Orlich, M., and Scholtissek, C., J. Gen. Virol. 44, 471 (1979).
22. Sugiura, A., and Ueda, M., Virology 101, 440 (1980).
23. Vallbracht, A., Scholtissek, C., Flehmig, B., and Gerth, H.-J., Virology 107, 452 (1980).
24. Klenk, H.-D., Rott, R., Orlich, M., and Blödorn, J., Virology 68, 426 (1975).

DIFFERENT SIALYLOLIGOSACCHARIDE RECEPTOR DETERMINANTS OF ANTIGENICALLY RELATED INFLUENZA VIRUS HEMAGGLUTININS

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ABSTRACT

Antigenically similar influenza virus substrains A/RI/5$^-$/57 (RI/5$^-$) and A/RI/5$^+$/57 (RI/5$^+$) appear to differ in their properties of elution from erythrocytes due to different specificities in the recognition of cell surface receptor determinants by their hemagglutinins. The RI/5$^-$ and RI/5$^+$ viruses preferentially bind to cell surface oligosaccharides containing the NeuAcα2,3Gal and NeuAcα2,6Gal sequences, respectively. Since the viral neuraminidases efficiently cleave the NeuAcα2,3Gal sequence but poorly cleave the NeuAcα2,6Gal linkage only the RI/5$^-$ virus can elute from the cells. Differences in receptor specificities are also observed between the H3 hemagglutinin of a human Hong Kong strain and the antigenically related equine Heq2 and avian Hav7 hemagglutinins suggested to be the genetic progenitors of the H3 hemagglutinin. These observations together suggest that different receptor specificities of influenza virus hemagglutinins can modulate to some degree certain functional properties of the viruses, and that receptor specificities among viral hemagglutinins can vary independently of their antigenic properties.

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INTRODUCTION

Numerous examples have been documented for functional differences among the influenza virus hemagglutinins, which bind to cell surface sialyloligosaccharides and the viral neuraminidases, which hydrolyze sialyloligosaccharides (1). Hemagglutinins have been found to differ both in their relative abilities to cause agglutination of the red cells of various species and in their sensitivity to various glycoprotein inhibitors of hemagglutination. Likewise, viral neuraminidases have been found to differ in their abilities to destroy the erythrocyte receptors of other viruses and to neutralize the activity of glycoprotein inhibitors of agglutination. However, there has been little information to describe in molecular terms the basis for these differences. In an attempt to gain some insight into these phenomenon, this report has examined the specificity of the interaction of several antigenically related influenza virus hemagglutinins and neuraminidases toward sialyloligosaccharides of defined sequence.

METHODS

Virus Propagation

Influenza virus strains A/RI/5$^+$/57 (H2N2) and A/RI/5$^-$/57 (H2N2) were gifts of Dr. Purnell Choppin and the avian strains A/Mallard/NY/6874/78 and A/Mallard/Memphis/546/78 were gifts of Dr. Robert G. Webster. Other strains including A/PR/8/34, Al/FM/1/47,A/Japan/305/57,A/Hong Kong/8/68, A/Equine 1/Prague 1/56 and A/Equine 2/Miami 1/63 were purchased from the American Type Culture Collection. All viruses were propagated in the allantoic cavity of 10-11 day old chick embryos. Except for the avian viruses all strains were partially purified from the harvested allantoic fluid by differential centrifugation.

Enzymatic Modification of Erythrocyte Oligosaccharides

Oligosaccharides of native human erythrocytes were modified as previously described (2). Asialo cells were prepared by treatment with Vibrio cholerae neuraminidase. Asialo cells were then resialylated by reaction with CMP-[^{14}C]NeuAc and either the β-galactosideα2,6, the αN-acetylgalactos-

aminideα2,6 or the β-galactosideα2,3 sialyltransferases to give the NeuAcα2,6Gal, NeuAcα2,6GalNAc or NeuAcα2,3Gal linkages, respectively. These three enzymes incorporated sialic acid into red cell glycoproteins to the extent of 59, 71, and 130 nmol sialic acid per ml packed cells. Hemagglutination titrations with these cells were performed in a microtiter system.

RESULTS

The Molecular Basis for the Differential Elution of the RI/5$^-$ and RI/5$^+$ Virus from Erythrocytes

The antigenically similar human influenza virus substrains A/RI/5$^+$/57 (RI/5$^+$) and A/RI/5$^-$/57 (RI/5$^-$), isolated from a single throat swab by Choppin and Tamm, exhibit several different biological properties (3). Although both viruses absorb to human erythrocytes, only the RI/5$^-$ virus will subsequently elute. Additionally, both infection of chick embryos and hemagglutination by the RI/5$^+$ virus is neutralized by horse serum glycoproteins, while the RI/5$^-$ virus is insensitive to such inhibition (3).

To understand the differential elution of the RI/5$^-$ and RI/5$^+$ viruses from erythrocytes, the specificities of their hemagglutinins and neuraminidases were examined using native human erythrocytes, neuraminidase treated (asialo) erythrocytes and asialo erythrocytes modified with purified sialyltransferases to contain the sequences NeuAcα2,6Gal,

TABLE I. THE INTERACTION OF RI/5$^-$ AND RI/5$^+$ INFLUENZA VIRUS WITH NATIVE, ASIALO AND RESIALYLATED ERYTHROCYTES

Erythrocyte Preparation	Sequence Restored	Viral Hemagglutination Titers[A]	
		RI/5$^-$	RI/5$^+$
Native	-	256	64
Asialo	-	0	0
Resialylated	NeuAcα2,6Gal	0	64
Resialylated	NeuAcα2,6GalNAc	0	0
Resialylated	NeuAcα2,3Gal	512	0

A) Hemagglutination is expressed as the maximum dilution required to give complete agglutination (0 = < 2).

NeuAcα2,6GalNAc or NeuAcα2,3Gal on cell surface oligosaccharides. The hemagglutinins of the two viruses had quite different specificities towards sialyloligosaccharide receptor determinants as shown in Table I. While neither virus agglutinated asialo cells, the RI/5$^-$ virus only agglutinated resialylated cells modified to contain the NeuAcα2,3Gal linkage, and the RI/5$^+$ virus only agglutinated cells containing the NeuAcα2,6Gal linkage.

The neuraminidases of both viruses were found to efficiently hydrolyze the NeuAcα2,3Gal linkage while the NeuAcα2, 6Gal linkage was a poor substrate. This specificity was observed both for the hydrolysis of the 3' and 6' isomers of sialyllactose and the sialyloligosaccharides of the resialylated erythrocytes (data not shown). These and supporting results have suggested that the different elution properties of the RI/5$^-$ and RI/5$^+$ viruses are primarily determined by differences in the receptor specificities of their viral hemagglutinins. The RI/5$^-$ virus binds to erythrocytes and its neuraminidase preferentially hydrolyzes the NeuAcα2,3Gal linkage, its receptor determinant, resulting in viral elution. The RI/5$^+$ virus once bound to the cells also preferentially hydrolyzes the NeuAcα2,3Gal linkage, but its receptor determinant, NeuAcα2,6Gal, is largely resistant to hydrolysis and therefore the virus fails to elute.

Comparison of Receptor Specificities of Human and Animal Influenza Viruses

It is of interest that the hemagglutinins of the RI/5$^-$ and RI/5$^+$ viruses have different receptor specificities in view of their antigenic similarity, especially since antigenic properties are often used to demonstrate the relatedness of different virus strains. This bears special relevance to the relationship of viruses isolated from different species (4). The specificities of a number of human, equine and avian viruses towards oligosaccharide receptors on erythrocytes are shown in Table II.

Avian and equine viruses only agglutinate derivatized cells containing the NeuAcα2,3Gal linkage while the human viruses also agglutinate cells containing the NeuAcα2,6Gal linkage. The H3 hemagglutinin of the human Hong Kong strains have been found to be remarkably similar antigenically and

chemically to the avian Hav7 and equine Heq2 hemagglutinins (4,6). Thus, the dramatic difference in receptor specificity of the H3 hemagglutinin and the animal virus hemagglutinins is particularly striking.

TABLE II. VIRAL AGGLUTINATION TITERS WITH ERYTHROCYTES DERIVATIZED TO CONTAIN DEFINED SIALYLOLIGOSACCHARIDES

Virus	Erythrocyte Preparation				
	Native	Asialo	NeuAcα2,3Gal	NeuAcα2,6Gal	NeuAcα2,6GalNAc
	(Agglutination Titer)				
Influenza A, Avian					
Mallard/NY (Hav7N2)	64	0	64	0	0
Duck/Memphis (Hav3Nav6)	16	0	16	0	0
Influenza A, Equine					
Eq Prague (Heq1Neq1)	2048	0	2048	0	0
Eq Miami (Heq2Neq2)	256	0	128	0	0
Influenza A, Human					
FM1 (H1N1)	512	0	512	128	0
PR-8 (H1N1)	1024	0	1024	1024	0
JAP/305 (H2N2)	1024	0	1024	1024	128
HK (H3N2)	256	0	0	256	0

DISCUSSION

Earlier studies suggested that the striking differences in the interactions of the RI/5$^-$ and RI/5$^+$ viruses with erythrocytes, and with glycoprotein inhibitors of hemagglutination resided in their hemagglutinins (3,7). As shown here the different elution properties of the two viruses from erythrocytes appear to be due to different specificities of the viral hemagglutinins for their cell surface receptor determinants. The RI/5$^-$ virus utilizes the oligosaccharide sequence NeuAcα2,3Gal as its receptor determinant, while the RI/5$^+$ virus binds preferentially to the sequence NeuAcα2,6Gal. Since both of the viral neuraminidases rapidly hydrolyze the NeuAcα2,3Gal linkage and only slowly hydrolyze the NeuAcα2,6Gal linkage, the RI/5$^-$ virus elutes from the cells while the RI/5$^+$ virus remains bound.

The different hemagglutinin specificities of the two viruses provide in part a molecular basis for the influenza virus receptor gradient (8) which ranked viruses according to their abilities to agglutinate human erythrocytes previously exposed to the viral neuraminidase of the same or another

virus. Since the neuraminidase of the RI/5$^-$ virus efficiently cleaves its own receptor determinant (NeuAcα2,3Gal) but not that of the RI/5$^+$ virus (NeuAcα2,6Gal), the RI/5$^-$ virus would be ranked above the RI/5$^+$ virus in the gradient. Indeed, treatment of native human erythrocytes with the RI/5$^-$ virus abolishes hemagglutination by that virus without effecting the agglutination titer of the RI/5$^+$ virus (not shown).

The avian influenza virus Hav7 hemagglutinin has been suggested to be the progenitor of the human influenza H3 hemagglutinin based on striking antigenic and structural similarities (4,6). Yet as shown in Table II, human and avian viruses carrying these hemagglutinins appear to have quite different specificities towards cell surface receptor determinants. In contrast, representative virus strains isolated from the same species have similar specificities towards the three sialyloligosaccharide sequences examined in this report. Although it is premature to suggest that the receptor specificity shift between the viruses bearing the avian Hav7 hemagglutinin and the human H3 hemagglutinin is more than coincidental, it will be of interest to explore the possibility that such a change has a functional purpose.

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REFERENCES

1. Hoyle, L., *in* "Virology Monographs" (S. Gard, C. Hallauer, and K.F. Meyer, eds.), Vol. 4, p. 1. Springer, New York, (1968).
2. Sadler, J.E., Paulson, J.C., and Hill, R.L., *J. Biol. Chem. 254,* 2112 (1979).
3. Choppin, P.W., and Tamm, I., *J. Exp. Med. 112,* 921 (1960).

4. Laver, W.G., and Webster, R.G., *Brit. Med. Bull. 35,* 29 (1979).
5. Frisch, A., and Neufeld, E.F., *Anal. Biochem. 95,* 222 (1979).
6. Scholtissek, C., Rohde, W., Von Hoyningen, V., and Rott, R., *Virology 87,* 13 (1978).
7. Compans, R.W., and Choppin, P.W., *in* "Comprehensive Virology" (H. Fraenkel-Conrat, and R.R. Wagner), Vol. 5, p. 179. Plenum Press, New York, (1975).
8. Stone, J.D., and Ada, G.L., *Brit. J. Exp. Path. 33,* 428 (1952).

Characterization of Influenza Virus Receptors on Host Cells

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ABSTRACT

It has been clearly established that influenza virions bind to soluble and erythrocyte-associated sialylated glycoproteins via the viral hemagglutinin and that the viral neuraminidase can release the virus from these substrates by removing terminal sialic acid residues from these glycoproteins. However, the relevance of these interactions to the initiation of infection is obscure. Although the viral neuraminidase is required for release of virus from these glycoproteins, we have shown that virions from which the neuraminidase was quantitatively removed by trypsin were fully infectious so long as the hemagglutinin spikes were not removed. Furthermore, addition of sialylated glycoproteins to trypsinized virus or covalent attachment of sialic acid residues to the hemagglutinin of the virus increased the infectivity of the preparations whereas it destroyed the hemagglutinating activity.

These apparently contradictory findings prompted us to attempt isolation of virus receptors from host cell membranes. Using Triton-solubilized cell membranes and affinity chromatography on asialofetuin-Sepharose, we isolated a membrane fraction which inhibited influenza virus binding and infectivity but had little effect on vesicular stomatitis virus. This fraction was eluted from the column by galactose. The inhibitory activity was quantitatively recovered in the chloroform following chloroform-methanol extraction. This chloroform-soluble fraction contained trace amounts of a poly-

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peptide which was resistant to degradation by pronase and by trypsin. When the chloroform extract was applied to a Florisil column, the inhibitory activity was eluted in the glycolipid fraction. It was not destroyed by proteases, lipase, phospholipases or by a variety of glycosidases, including neuraminidases. It was, however, destroyed by β-galactosidase from bovine liver; this destruction was prevented by galactonolactone, a specific inhibitor of β-galactosidase. The inhibitory component appears to be a glycolipid with terminal galactose residues. The results suggest that, during the initial stages of infection, influenza virions interact with glycolipids in the plasma membranes and that these glycolipids must have terminal galactose residues in order for this interaction to occur.

I. INTRODUCTION

It has been known for nearly forty years that influenza virions bind to sialic acid containing molecules on erythrocytes and, as a consequence, cause agglutination of these cells (1,2). Within a few years of this observation, the essential features of this virion-induced hemagglutination had been determined and the hemagglutination reaction was established as a valuable tool for virus quantitation and for the detection of antibodies against the viral hemagglutinin. Early work also indicated that soluble sialylated glycoproteins inhibited hemagglutination (3,4) and it has been widely assumed that virions fail to bind to erythrocytes in the presence of these inhibitors. In recent years interest in the interaction of influenza virions with erythrocytes has been revived, this time as a model for studying the properties of receptors for the influenza viruses. Much of the work which has been carried out involves the isolation of soluble sialylated glycoproteins from erythrocyte membranes (5,6). In addition, lipid bilayers of defined chemical composition have been constructed. Again, it has been conclusively demonstrated that sialic acid residues on either glycoproteins or glycolipids will mediate influenza virion bindings so long as a sufficient number of these residues are present (7).

However, the relevance of these reactions to the infectious process remains obscure because erythrocytes do not synthesize influenza virions and because a variety of experimental observations indicate that interactions between the virus and host cells do not necessarily mimic those between the virus and erythrocytes. We have therefore undertaken a number of different lines of investigations in an attempt to

determine which viral envelope components and which host cell membrane components are involved in the initiation of infection. This report summarizes our earlier work in which we have studied the effects of various modifications of influenza virions on their ability to initiate infection. It also describes our recent attempts at obtaining solubilized receptor components from host cell membranes.

II. RESULTS

A. Modification of Influenza Virions Surface Components

The WSN strain of influenza A has been used in most of the experiments to be described. We have studied the effects of three treatments on the infectivity of this strain of virus and on its ability to bind to erythrocytes and to host cells. As shown in Table 1, Treatment 1, we have quantitatively removed the neuraminidase from the surface of the virions with trypsin without reducing their ability to initiate infection. With the WSN strain, this treatment consistently results in a slight increase in plaque titer without an increase in association of virions with host cells or erythrocytes.

We have also shown that we can produce infectious but non-hemagglutinating virions by covalently attaching sialic acid residues to the surface of influenza virions (Treatments 2 and 3 in Table 1). As previously described in the references cited, the sialic acid residues were covalently linked to terminal galactose residues on the hemagglutinin and could be at least partially removed by the viral neuraminidase when incubated under conditions optimum for the viral enzyme activity. Virus preparations with about 2750 sialic acid residues per particle remained fully infectious despite the fact that the isoelectric point of the virions had been reduced from about 6.5 to below 4.0. As shown in Table 1, Treatments 2 and 3, the amount of stimulation of infectivity depended on whether the virions were intact or trypsinized and on the type of cell used for the plaque assay. The experiments clearly showed that they could initiate infection at least as well as unsialylated virions.

Sialylated soluble glycoproteins have previously been shown to be potent inhibitors of influenza virus induced hemagglutination. With some virus strains the infectivity was also destroyed whereas with others, infection could occur in the presence of these inhibitors. Since the infectivity

Table 1. Interaction of Influenza Virions with Host Cells and Erythrocytes after Various Treatments

Treatment of Virus before Assay	Biological Activities				Association of Radioactive Virus with Cells			Ref.
	PFU on		HAU	N'ase	CEF	MDBK	RBC	
	CEF	MDBK						
	(times control)				(times control)			
1. Trypsinized	1.7	1.7	1.0	0[a]	1.0	1.0	1.0	8
2. Trypsinized and sialylated	2.5	2.4	0.01	0[a]	1.0[b]	1.0[b]	1.0[b]	9,10,11
3. Sialylated	3.6	10.0	0.01	1.0	0.95[b]	1.0	0.98	9,10,11
4. Fetuin added	3.7	6.0	0.05	-[c]	-	0.62[b]	0.98	12
5. Asialofetuin added	-	1.0	1.0	-	-	-	-	12
6. Trypsinized, fetuin added	-	11.0	0.04	-	-	0.51[b]	0.93	12
7. Trypsinized, asialofetuin added	-	2.8	1.0	-	-	-	-	12

[a]Less that 0.02% of control values
[b]unpublished data
[c]not done

of virions of the WSN strain was not destroyed by removal of the neuraminidase, we were able to reinvestigate the effects of soluble glycoproteins on the infectivity of purified virus under conditions in which the virus could not release itself from these molecules by removing their sialic acid residues. As shown in Table 1, Treatments 4 and 6, plaque production was enhanced when either intact or neuraminidase-free virions were incubated with the sialylated glycoprotein, fetuin, prior to assay. In addition, we found that the extent of this enhancement was dependent on the type of cell monolayers used for plaque assay (Treatment 4) and on whether the virion preparation had been trypsinized prior to treatment with fetuin (Treatment 4 versus 6). Asialofetuin was found to have little enhancing activity (Treatments 5 and 7), again confirming the importance of sialic acid residues in this reaction.

The experiments summarized in Table 1 clearly show that influenza virions with sialic acid residues on their surfaces are, at the very least, as capable of initiating infection as are virions which are free of these residues. It must therefore be concluded that they prevent neither adsorption nor penetration. Their ability to enhance plaque production can be due, in part, to the formation of infectious units by the aggregation of incomplete particles in the virus preparation. However, such an explanation does not adequately explain the differences in degree of enhancement seen between the two host cells or between trypsinized and intact virus. Thus, sialic acid residues on the virion either promote more effective binding or they facilitate penetration of the virus through the cell membrane.

B. Modification of Cell Surfaces by Neuraminidase Treatment

The experiments just described suggested that cell membrane components other than sialylated molecules may be important in the initial steps of infection. They led us to reinvestigate the effect of neuraminidase treatment of cells on their susceptibility to influenza virus. MDBK cells were treated with neuraminidase and immediately tested for their ability to support the production of both influenza virus and vesicular stomatitis virus (VSV) plaques. Under these conditions a reduction in influenza virus plaque number without a concomitant reduction in VSV plaques would support the idea that sialic acid containing receptors were needed for influenza virus infection but not for VSV. As shown in Figure 1, this treatment reduced influenza virus plaque production by about 80% whereas it slightly increased the plating efficiency of VSV. This latter result virtually eliminates the possibil-

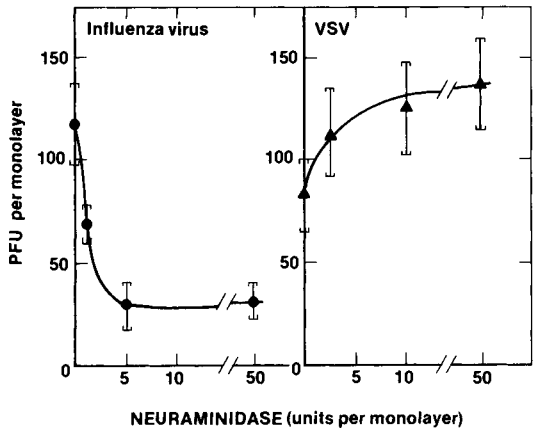

FIGURE 1. Effect on plaque production of pretreatment of MDBK monolayers with neuraminidase. MDBK monolayers in 5 cm plastic dishes were treated with V. cholera neuraminidase for one hour at the concentrations indicated. The WSN strain of influenza virus and VSV were then applied to separate plates. After one hour at room temperature, the monolayers were overlayed and incubated as previously described (18). Brackets indicate two standard deviations from mean counts from duplicate plates.

ity that neuraminidase treatment reduces influenza virus plaque production by damaging the synthetic capacities of the cells.

Although there are other explanations for these results, the experiments strongly suggest that removal of sialic acid residues from host cells reduces influenza virus plaque formation by reducing the likelihood of virus attachment. However, as shown in Figure 1, even after treatment with large amounts of neuraminidase, approximately one fifth of the cells can still be infected by a single infectious unit of influenza virus. The basis for this residual susceptibility remains to be determined.

The observations summarized thus far suggested to us that, while sialic acid residues may be largely responsible for the initial attachment of virions to host cells, interaction of the bound virions with other membrane components is required for effective binding and penetration. We postulated further that a second receptor component in the membrane could interact

with a specific region of the cleaved hemagglutinin (13,14) thereby promoting penetration rather than simple binding. Experiments designed to detect this second receptor substance make up the remainder of this report.

C. Isolation of a Cell Membrane Component Involved in the Initial Stages of Infection

Our attempts at isolating influenza virus receptors from host cells were based on the premise that, when solubilized, these cell membrane components would interfere with the initial steps of infection. Since there is now evidence that some unrelated viruses can use the same receptors (15) we further postulated that the virus-receptor interaction did not necessarily require a high degree of structural specificity and that the virus might bind to a variety of membrane components which had high affinities for asialoglycoproteins. Accordingly, asialofetuin was used in place of the viral hemagglutinin to construct an affinity column. In addition, we reasoned that we should reduce hydrophobic interactions which would lead to aggregation of the solubilized membrane components by keeping a detergent present throughout the separation procedure.

Sepharose with covalently attached asialofetuin was prepared as described by Hudgin et al. (16) and treated with 1% bovine serum albumin to saturate nonspecific binding sites. A column with a bed volume of 2.0 ml was then equilibrated with the loading buffer (10 mM Tris (pH 7.8) containing 50 mM $CaCl_2$, 1.25 M NaCl and 0.5% Triton X-100). Triton extracts of cell membranes from about 10^8 cells were prepared for application to the column as follows. Following multiple freeze-thaw cycles, the membrane pellets were collected by centrifugation at 104,000 x g, washed, and solubilized in 0.01 M Tris, pH 7.8 containing 0.4 M KCl and 1.0% Triton X-100. Following centrifugation of this extract, a clear supernatant fraction was obtained. This was applied to the affinity column in the loading buffer and, after washing the column to remove unadsorbed components, elution was carried out first with 20 mM ammonium acetate (pH 6.0) containing 1.25 M NaCl and 0.5% Triton, and then with the same buffer containing 0.2 M galactose. The two eluates were dialyzed extensively against 10 mM Tris HCl, pH 7.0, and concentrated by exposing the dialysis bags to Sephadex G-25.

The inhibitor activities of the various fractions from a typical preparation are presented in Table 2. Inhibitor activity was measured by mixing different-sized aliquots of

Table 2. Solubilization and Purification of an Inhibitor of Influenza Virus from MDBK Cell Membranes

	Fractions	Inhibitor Units per Fraction
I.	MDBK cell membranes	144
II.	Triton extract	4872
III.	Material not bound to asialofetuin	< 800
IV.	pH 6.0 eluate	9×10^4
V.	Galactose eluate	1.4×10^5
IV. + V.	Total units eluted	2.3×10^5

Inhibitor unit equals the amount required to reduce plaque member by 50%.

each fraction with influenza virus diluted to approximately 500 PFU per ml. The size of the aliquots which could be tested was in some cases limited by the Triton concentration of the fractions. The amount of Triton in each was determined by red cell lysis and was kept below that which inactivates influenza virus (17). After one hour at room temperature the mixtures were applied in duplicate to confluent MDBK monolayers. Plaque assays were carried out as described (18). One inhibitor unit was taken as that amount which reduced plaque production by 50%; the number of inhibitor units was calculated from semilog plots of residual plaque number vs. inhibitor volume. As shown in Table 2, Triton extraction drastically increases the total number of inhibitor units. Another large increase in activity was obtained in Fractions IV and V, those fractions eluted from asialofetuin by pH changes and the addition of galactose. Fraction III contained most of the protein applied to the column and, when added back to Fraction V, prevented inhibition of plaque formation. We have concluded that Fraction III contains membrane components which bind to the virus inhibitor and prevent its action.

Although Fractions IV and V contained comparable amounts of inhibitor, Fraction IV contained a number of proteins as

determined by SDS-polyacrylamide gel electrophoresis whereas only trace amounts of proteins were found in Fraction V. Fraction V was therefore used for further characterization. As shown in Table 3, when one volume of Fraction V was extracted with 2 volumes of $CHCl_3$:CH_3OH (2:1), its inhibitory activity was quantitatively recovered in the $CHCl_3$ layer. Treating Fraction V with various enzymes or with heat at high and low pH showed the inhibitory component to be stable to all treatments except β-galactosidase from bovine liver and heating at low pH.

No loss of inhibitory activity was observed with neuraminidase treatments suggesting that the inhibitor is either not sialylated, that sialic acid residues were inaccessible to the enzyme, or that they were not required for activity.

Table 3. Effect of Various Treatments on the Inhibitory Activity of Fraction V

Incubation Mixture	PFU/plate
Virus alone	390
Virus + Fraction V	40
Virus + $CHCl_3$ phase after $CHCl_3$:CH_3OH extraction of Fraction V	37
Virus + aqueous phase after extraction	405
Virus + treated Fraction V	
1.5 units β-galactosidase, 48 hrs, 37°	408
100°, 1 hr	45
0.1 N HCl, 100°, 1 hr	438
0.1 N NaOH, 100°, 1 hr	35

Virus and Fraction V were incubated for one hour at room temperature before plaque assay as previously described (18). Trypsin, Pronase, Lipase, Phospholipase C and A_2, β-N-acetyl glucosaminidase, β-glucosidase and neuraminidase had no effect on inhibitor activity.

That the destruction of inhibitor activity by β-galactosidase is indeed due to enzymatic cleavage is shown in Table 4. After 48 hours of incubation with β-galactosidase, 95% of the initial activity was destroyed. This destruction was prevented by a specific inhibitor of β-galactosidase. It can therefore be concluded that terminal galactose residues are a structural requirement for this activity. This conclusion is supported by recent data indicating that Fraction V contains tritium when it is prepared from cells which have been surface labeled by treatment with galactose oxidase and tritiated borohydride.

Based on data like that presented in Tables 3 and 4 we have tentatively concluded that the inhibitory activity resides either in glycolipids, in proteins which quantitatively extract into $CHCl_3$, or in a complex structure which contains both moieties. We have found that a protein of approximately 10,000 daltons is present in the chloroform soluble material from Fraction V even when extraction is carried out after pronase treatment. Whether this protein is a contaminant or an integral part of the solubilized inhibitor remains to be determined. Since the inhibitory component is available to β-galactosidase and is destroyed by this enzyme whereas the protein is not available to proteases, glycolipids with terminal galactoses appear to be an essential part of the inhibitor.

Table 4. Destruction of Inhibitor Activity by β-Galactosidase

	Inhibitor activity after 48 hours at 37° (% of initial units)
Inhibitor alone	110
Inhibitor + β-galactosidase	5
Inhibitor + β-galactosidase + D-galactono-1,4-lactone	100

β-galactosidase (1.5 units) from bovine liver was added to 100 units of inhibitor in 10 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 20 mM NaCl and 1 mM mercaptoethanol. The final concentration of D-galactono-1,4-lactone was 10 mM. The reaction volume in all cases was 0.2 ml.

Thus far we have not identified the glycolipids present in our extracts. All inhibitor preparations obtained by the procedure just described contain Triton which interferes with this identification. We have therefore attempted to obtain the same material by direct extraction of membranes with $CHCl_3$:CH_3OH, followed by chromatography on magnesia-silicate (Florisil). In this procedure, lipid components are separated by elution with solvents of increasing polarity (19). Although the inhibitor activity is recovered in the glycolipid fraction, material prepared in this way has greatly reduced total and specific activity (0.6 IU/μg glycosphingolipid) as compared to that of the chloroform soluble components of Fraction V (>12 IU/μg glycosphingolipid). When Fraction V is applied to a Florisil column, its inhibitory activity is also greatly reduced with that remaining eluting in the glycolipid fraction. Investigations are underway to determine whether trace amounts of detergents will augment inhibitor activity by increasing its effective concentration.

D. Effects of Triton-Solubilized Inhibitor on Influenza Virus and on VSV.

The remainder of this report deals with the biological effects of the chloroform soluble components of Fraction V. The experiments to be described focus on the interaction of influenza virions with host cells. However, prior to testing its effect on virus host cell interactions, we tested its effect on the integrity of the virus particles. Inhibitor was incubated with ^{32}P-labeled virions and the mixture was applied to sucrose gradients. The sedimentation properties of treated and untreated control virus were identical at concentrations of inhibitor ten-fold greater than that needed to obtain 90% inhibition of plaque production.

Figure 2 shows the effect of increasing inhibitor concentration on influenza virus plaque formation. As the concentrations of inhibitor is increased in increments up to approximately 4 units, 50% reduction in residual infectivity is observed for each unit added.

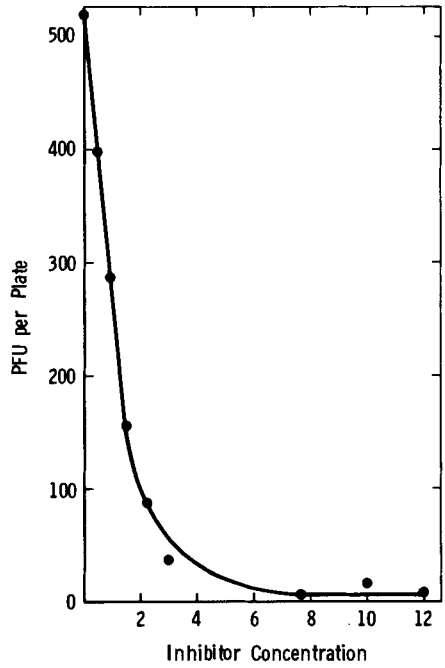

FIGURE 2. Inhibition of influenza virus plaque production by solubilized membrane components. Influenza virus diluted to approximately 1000 PFU per ml in phosphate buffer containing gelatin (18) was incubated for one hour at room temperature with the chloroform-soluble components of Fraction V at the concentrations indicated. The mixtures were then plated in duplicate on MDBK monolayers, adsorbed for one hour, and overlayed as previously described (18). Inhibitor concentration is in units per sample.

The specificity of the isolated inhibitor for influenza virus is shown in Figure 3. Whereas the plating efficiency of influenza virus is reduced by increasing amounts of inhibitor, that of VSV is not. We, therefore, have concluded that this material has little effect on VSV adsorption and/or penetration.

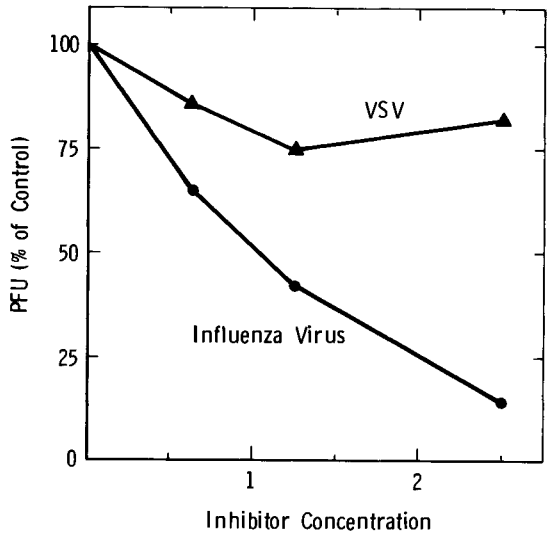

FIGURE 3. Effects of inhibitor on influenza virus and on VSV. The experiments were carried out as described for Figure 2. The two viruses were applied to separate plates. Control plates contained approximately 200 plaques of each virus.

As shown in Table 5, pretreatment with inhibitor reduced the association of virus with MDBK cells by 30 to 46%. Greatest inhibition was observed with the shortest adsorption time suggesting that prolonged virus cell contact permitted a small amount of reversal of inhibition.

Table 5. Effect of Inhibitor on Association of Virions with MDBK Cells

Time after addition of virus to cells	Virus associated with cells		
	Virus alone	Virus + inhibitor	Virus + inhibitor / Virus alone
(minutes)	(cpm per monolayer)		
15	325	100	0.30
30	460	150	0.33
60	570	255	0.45
120	570	260	0.46

Influenza virus was grown in MDBK cells in the presence of $^{32}PO_4$. Virus yields harvested at 40 hours were purified as described by Pons and Hirst (20). Fifteen units of inhibitor were added to 3000 HAU of purified virus and incubated at room temperature for one hour. 100 HAU of virus alone or with inhibitor were added to MDBK monolayers containing 10^6 cells. At the times indicated the amount of virus associated with monolayers was measured as previously described (11).

The effect of varying inhibitor concentration on association of virus with host cells is shown in Figure 4. Although increasing inhibitor concentration reduced the amount of virus associated with MDBK cells, the dose response shown here is significantly different from that shown in Figure 2. The basis for this difference is under investigation. Current results suggest that much lower doses of inhibitor are required to prevent infection than to inhibit attachment of virions to the cell surface.

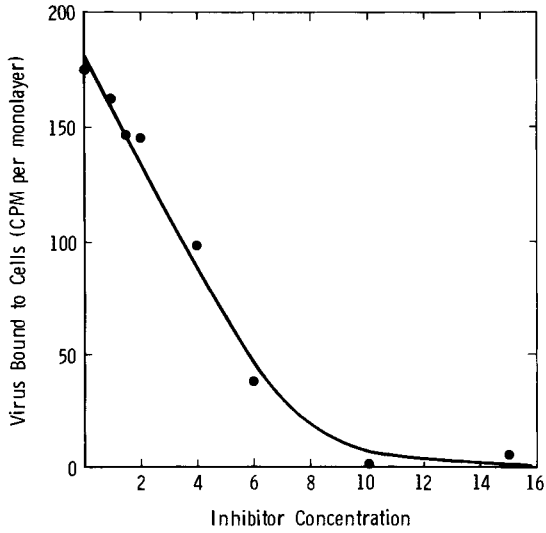

FIGURE 4. Effects of various concentrations of inhibitor on association of influenza virus with MDBK cells. The experiment was carried out as described for Table 5, except that various concentrations of inhibitor were used as indicated and the amount of virus associated with the cells was measured after one hour at room temperature.

III. DISCUSSION

The aim of these experiments was to identify those components on host cell membranes with which influenza virus interacts in the process of initiating infection. The experimental approach which we adopted was to purify components which would prevent plaque formation when removed from the membrane. Sialylated glycoproteins would appear to be important in this regard since influenza virions are known to bind to sialic acid residues on glycoproteins and glycolipids, and removal of these residues from MDBK cells decreases the plating efficiency of this virus. However, our experiments have shown that soluble glycoproteins fail to inhibit the infectivity of the WSN strain even when the virus is devoid of neuraminidase activity. Thus, by requiring that plaque formation be inhibited by the solubilized membrane components, we greatly de-

creased the possibility of isolating a sialylated glycoprotein. As a consequence, we have isolated from MDBK cell membranes a fraction which appears to be directly involved in the process of infection but vastly different from the solubilized inhibitors of hemagglutination which have been previously characterized. The material described here shows increased inhibitory activity as it is purified. It is soluble in chloroform and presumably requires low levels of detergent to keep it in solution when removed from the membrane lipid bilayer. It inhibits plaque formation at very low concentrations and its antiviral activity is dependent on its having terminal galactose residues. Although the chemical nature of this material remains to be determined, a variety of pieces of data suggest that it consists of glycolipids either alone or in complex with a small hydrophobic protein.

The exact role of this material in the infectious process is at present also not known. Since it inhibits influenza virus but not VSV, it is likely that it provides a function specifically needed by influenza virus. Although it does reduce association of the virus with cells, it inhibits infection at much lower concentrations than it prevents binding, suggesting that virus particles attached to the plasma membrane fail to penetrate in the presence of the solubilized membrane component.

The interplay of sialylated membrane components and our chloroform soluble material in the infectious process needs to be further investigated. At present, it appears likely that sialic acid containing molecules may be important for the initial binding of the virus to the cell, but that this binding is not sufficient for initiation of infection. In this regard, it is important to note that influenza virions are not infectious unless the viral hemagglutinin is cleaved (13, 14) in a specific fashion which generates two disulfide linked chains, one of which (HA2) has a hydrophobic N-terminal region. Interaction of this part of the hemagglutinin structure with the membrane may be essential for penetration. Alternatively, interactions with hydrophobic membrane components, like the material described here, could cause configurational changes in the cleaved hemagglutinin which would permit a close approach of the viral lipid bilayer to the cell membrane.

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REFERENCES

1. Hirst, G.K. (1941). Science 94, 22.
2. McClelland, L., and Hare, R. (1941). Can. Pub. Health J. 32, 530.
3. Hirst, G.K. (1942). J. Exp. Med. 76, 195.
4. Francis, T. (1947). J. Exp. Med. 85, 1.
5. Winzler, R.J. (1969). In "Red Cell Membrane, Structure and Function" (G.A. Jamieson and T.J. Greenwald, eds). p. 157 Lippincott, Philadelphia, Pennsylvania.
6. Marchesi, V.T., Furthmayr, H., and Tomita, M. (1976). In "Cell Membrane Receptors for Viruses, Antigens and Antibodies, Polypeptide Hormones, and Small Molecules" (R.F. Beers, Jr. and E.G. Bassett, eds) Raven Press, New York.
7. Haywood, A.M. (1974). J. Mol. Biol. 83, 427.
8. Schulze, I.T. (1970). Virology 42, 890-904.
9. Schulze, I.T. (1975). In "Negative Strand Viruses (R.D. Barry and B.W.T. Mahy, eds) Vol. I, p. 161, Academic Press, New York.
10. Schulze, I.T. (1975). In "The Influenza Viruses and Influenza" (E.D. Kilbourne, ed), p. 53, Academic Press, New York.
11. Lakshmi, M.V., and Schulze, I.T. (1978). Virology 88, 314.
12. Lakshmi, M.V., Der, C.L., and Schulze, I.T. (1978). Topics in Infec. Diseases 3, 101.
13. Lazarowitz, S.G., Goldberg, A.R., and Choppin, P.W. (1973). Virology 56, 172.
14. Klenk, H.-D., Rott, R., Orlich, M., and Blödorn, J. (1975). Virology 68, 426.
15. Lonberg-Holm, K., Crowell, R.L., and Phillipson, L. (1976). Nature 259, 679.
16. Hudgin, R.L., Pricer, W.E., Ashwell, G., Stockert, R.J., and Morell, G.A. (1974). J. Biol. Chem. 249, 5536.
17. Schlesinger, S., Sly, W.S., and Schulze, I.T. (1978). Virology 89, 409.
18. Noronha-Blob, L., and Schulze, I.T. (1976). Virology 61, 314.
19. Saito, T., and Hakomori, S. (1971). J. Lipid Res. 257, 12.
20. Pons, M., and Hirst, G.K. (1968). Virology 34, 385.

HOST DEFENSE AGAINST INFLUENZA

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The purpose of this article is to review recent research, primarily from our own laboratory, which better defines the specific host defense mechanisms involved in the prevention of and recovery from influenza. Ultimately our goal is to understand the relative roles of systemic cell mediated immunity (CMI), systemic antibody, local CMI, and local antibody in the prevention of both upper (URT) and lower respiratory tract (lung) influenza infection and in the recovery from both upper and lower respiratory tract influenza infection. These relationships can best be summarized in Figure 1. An alternate way of stating our

FIGURE 1

	Prevention		Recovery	
	URT	Lung	URT	Lung
Systemic				
Antibody	____	____	____	____
CMI	____	____	____	____
Local				
Antibody	____		____	
CMI	____		____	

overall goal is to say that we hope to fill in all the blanks in Figure 1. Our definitions of URT vs. lung are

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perhaps misleading and might be better broken down into airways with ciliated respiratory epithelium, (i.e., nose, trachea, bronchi and bronchioles) vs. alveoli. Because the alveolus is in such intimate association with the systemic circulation, we have chosen to ignore local immunity of the alveolus, if such exists.

This article will deal primarily with animal research, because it is very difficult to critically evaluate host defense mechanisms in man. The vast majority of research dealing with human influenza can only establish correlations e.g. between serum or local antibody and immunity to infection, but cannot establish a cause and effect relationship. Fundamentally, there are only two ways to establish a cause and effect relationship between a component of the immune system and prevention of, or recovery from, an infection. The first, selective addition of one immune component, was originally utilized by Behring and Kitasato (1890) when they were able to confer immunity on an animal by passive transfer of serum antibody. The second approach is to selectively ablate one component of the immune system and show that the non-immune animal cannot develop immunity or that the previously immune animal has lost that immunity. In man, it is obviously unethical to utilize the second approach but studies of immunodeficient children are adding to our understanding. To our knowledge, the first approach has not been utilized in man. Although we are primarily interested in human host defense mechanisms, we are thus forced to rely predominantly on animal models.

PREVENTION OF URT INFLUENZA INFECTION

There have been few studies of URT infection, relative to the large number of studies of influenza pneumonia. In the ferret, Small *et al.* (1976) showed that passive administration of ferret anti-influenza antisera in doses sufficient to give hemagglutination inhibition (HI) titers equal to or exceeding those of convalescent ferrets did not decrease either the amount or the duration of virus shedding when ferrets were challenged intranasally with homologus influenza virus. Interestingly enough, the passive antibody did suppress the subsequent production of antibody by the ferret. On rechallenge, most ferrets were immune in the absence of detectable serum HI antibody, again suggesting that serum antibody is irrelevant to prevention of URT influenza infection.

Another set of experiments (Barber and Small, 1978)

were performed by surgically creating a tracheal pouch which was anatomically isolated from the remainder of the URT and then infecting the pouch or the nose with influenza virus. Infection of either site did not spread to the other site, but did stimulate similar amounts of serum HI antibody and presumably comparable amounts of CMI although the latter was not measured. Three weeks after infection of the pouch, the ferrets were challenged in both the pouch and the nose with the same dose of influenza virus. No virus was subsequently shed from the pouch but as much virus was shed from the nose on day 1 as from control ferrets infected intranasally. Nasal shedding on day 3 was markedly reduced and by day 5 had stopped in contrast to the control group which shed for 7 days. Prior infection of the nose provided solid immunity against reinfection of the nose. Thus the prior pouch infection, which stimulated ample antibody and presumably CMI, did not prevent the URT infection but did promote more rapid recovery. As shown in Fig. 2, we concluded that

FIGURE 2

	Prevention		Recovery	
	URT	Lung	URT	Lung
Systemic				
Antibody	No	____	____	____
CMI	No	____	____	____
Local				
Antibody	Yes		____	
CMI			____	

neither systemic antibody nor CMI play a role in prevention of influenza infection, but that local immunity (Ab and/or CMI) is essential. These results are at variance with the conclusions presented by Dr. Couch based on his studies in man. This will be discussed below.

PREVENTION OF VIRAL PNEUMONIA

The passive antibody and tracheal pouch experiments were in potential conflict with a long established and well confirmed (Loosli *et al.*, 1953; Schulman *et al.*, 1968; Virelizier, 1975) set of observations in mice, wherein several laboratories had shown that passively administrated serum antibody prevented viral pneumonia. This suggested either that mice differed from ferrets or that URT infection was different from lung infection. The use of scanning

electron microscopy (SEM) to evaluate tracheal pathology in the mouse (Ramphal et al., 1979a) gave us a method for differentiating between these two possibilities (Ramphal et al., 1979b). Three groups of mice were studied: 1) mice convalescent from influenza infection, 2) mice passively immunized with normal ferret serum and 3) mice passively immunized with immune ferret serum. The convalescent group was solidly immune. The control group developed both URT infection and viral pneumonia and died. The passive antibody group developed URT infection, as demonstrated by tracheal desquamation, but did not develop viral pneumonia and they all lived. Thus these data confirmed our previous observation that serum antibody does not prevent URT infection, and also confirmed the three prior studies which showed that serum antibody does prevent viral pneumonia.

The question arises as to whether CMI can prevent lung infection. Theoretically one would not expect T cells to be able to neutralize virus in nasal or pulmonary secretion, but rather that CMI would be involved in destroying infected cells. By definition, this would then be recovery and not prevention. Experimentally, Yap and Ada (1979) gave anti influenza T cells to mice and showed that this treatment did not prevent subsequent infection. However, the T cells, as discussed below, did enhance recovery. As summarized in Figure 3, this limited theoretical and experimental work

FIGURE 3

	Prevention		Recovery	
	URT	Lung	URT	Lung
Systemic				
Antibody	No	Yes	____	____
CMI	No	No	____	____
Local				
Antibody	Yes		____	
CMI			____	

suggests that CMI is irrelevant to prevention of influenza pneumonia. There is general agreement that serum antibody prevents influenza pneumonia.

RECOVERY FROM INFLUENZA INFECTION

The animal of choice in which to study recovery from influenza infection is the nude mouse, since this animal

does not recover but sheds virus for weeks (Yap et al., 1979; Wells et al., 1981; Yetter et al., 1981). This enables one to selectively reconstitute the immune system and see what components thereby lead to recovery. Wells et al. (1981) have reconstituted the nude mouse with T cells stimulated in a variety of ways and shown that the reconstituted mouse recovers, but that recovery is most rapid and complete when T cells are used which have enhanced cytotoxic activity. T cells that promote antibody formation are not nearly as effective at promoting recovery. They conclude, "that cytotoxic T lymphocytes are associated with recovery of mice with influenza pneumonia, but immune spleen cells that enhance antibody responses are not." The conclusion that cytotoxic T cells are required for recovery is consistent with the observation of Yap and Ada (1978) who showed that cytotoxic T lymphocytes promoted recovery in normal mice as evidenced by decreased virus shedding from lungs. These elegant experiments utilized both H-2 restriction and anti Ly antisera to demonstrate that the cytotoxic T cell was required.

The conclusions of Wells et al. (1981) concerning antibody are in conflict with our own observations (Yetter et al., 1981) wherein we gave passive antisera to nude mice already infected with influenza virus and observed that they temporarily stopped shedding virus from lungs, trachea and nose and regenerated their tracheal epithelium. However, a few weeks later, when they no longer had detectable antibody, they resumed shedding virus from nose, trachea, and lung, and again desquamated their tracheas. Thus it appears to us that serum antibody can promote recovery, but that it is not sufficient to cure the animal. The crucial experiment of reconstituting the nude mouse with helper T cells has not yet been done. Whether constant antibody production could chronically suppress virus shedding and thereby promote recovery or alternatively just neutralize virus and lead to massive amounts of antibody antigen complex remains to be seen.

Likewise, the reconstitution of nude mice with cytotoxic T cells is yet to be done. Reconstitution of nude mice with lymphocytes and of normal mice with cytotoxic T cells both strongly suggest that cytotoxic T cells will lead to the recovery from viral pneumonia. Presumably a similar mechanism will apply to the URT. Fig. 4 summarizes our current understanding of recovery. At present there is no data to suggest that the immunologic recovery mechanisms for the URT differ from those of the lung. Serum antibody is not

FIGURE 4

	Prevention		Recovery	
	URT	Lung	URT	Lung
Systemic				
Antibody	No	Yes	Temp*	Temp*
CMI	No	No	Prob+	Prob+
Local				
Antibody	Yes		?	
CMI			?	

*Temp = Temporary recovery
+Prob = Probably

sufficient to promote permanent recovery of lungs or URT. There is conflict between two laboratories as to how much it helps in recovery. Cytotoxic T cells will lead to recovery of the lungs and probably the URT. Currently it is not possible to differentiate between systemic and local CMI in the recovery process. Virtually nothing is known about the role of local immunity in recovery of the URT.

DISCUSSION

As presented elsewhere in the volume (Couch et al., 1981), studies on human volunteers show a correlation between prevention of URT influenza infection and serum antibody but not between such prevention and IgA local antibody. As pointed out earlier in this paper, Dr. Couch's correlations cannot be proven to be a cause and effect relationship. However, his data are obviously in conflict with our own findings in ferrets and mice. There are several possible explanations for this discrepancy. Dr. Couch's studies use lower challenge doses, since he must avoid endangering his human volunteers while our own studies use relatively larger doses. Since we do not know either how the influenza virus spreads in nature (aerosol vs. fomite) nor the dose of virus transmitted in the average infection, it is unclear which of the dose ranges is more physiological. A second difference is that Dr. Couch waits 1 to 4 years to rechallenge his volunteers, while we only wait weeks to months to rechallenge our animals. His waiting periods are clearly appropriate for man. It is unclear what time period in mice or ferrets would correspond to 1 or more years in man. What is clear is that more studies are needed to determine whether the host defense mechanism summarized in Fig. 4 are dependent on the dose of challenge virus and

whether they change with time. In any case, the only way to unambiguously establish whether serum antibody can prevent human influenza infection is to passively transfer antibody to susceptible people and determine whether it protects them from subsequent virus challenge. In adult mice and ferrets it does not prevent URT infection but does prevent viral pneumonia.

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REFERENCES

Barber, W.H., and Small, P.A., Jr. (1978) Infect. and Immun. 21, 221.

Behring, E., and Kitasato. (1890) Deutsche Med. Wchnschr. XVI, 1113.

Couch, R.B., Kasel, J.A., and Cate, T.R. (1981) In "Genetic Variation Among Influenza Viruses - ICN-UCLA Symposia on Molecular and Cellular Biology" (D. Nayak and C.F. Fox, eds.), Academic Press, New York.

Loosli, C.G., Hamre, D., and Berlin, B.S. (1953) Trans. Assoc. Am. Phys. 66, 222.

Ramphal, R., Cogliano, R.C., Shands, J.W., Jr., and Small, P.A., Jr. (1979) Infet. and Immun. 25, 992.

Ramphal, R., Fischlschweiger, W., Shands, J.W., and Small, P.A., Jr. (1979) Am. Rev. Resp. Dis. 120, 1313.

Schulman, J.S., Khakpour, M., and Kilbourne, E.D. (1968) J. Virol. 2, 778.

Small, P.A., Jr., Waldman, R.H., Bruno, J.C., and Gifford, G.E. (1976) Infect. and Immun. 13, 417.

Virelizier, J.L. (1975) J. Immunol. 115, 434.

Wells, M.A., Ennis, F.A., and Albrecht, P. (1981) J. Immunol. 126, 1042.

Yap, K.L., and Ada, G.L. (1978) Nature 273, 238.

Yap, K.L., and Ada, G.L. (1979) Scand. J. Immunol. 10, 325.

Yap, K.L., Braciale, T.J., and Ada, G.L. (1979) Cell. Immunol. 43, 341.

Yetter, R.A., Cogliano, R.E., Ramphal, R., Gifford, G.E., and Small, P.A., Jr. Submitted.

Ir GENE CONTROL OF THE CYTOLYTIC T CELL RESPONSE TO INFLUENZA VIRUS

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I. ABSTRACT

We have investigated the genetic control of the cytolytic T lymphocyte (CTL) response to influenza virus in mice. Although most strains respond equally to H-2K or H-2D antigens in association with influenza virus, we observed that one strain (C57BL/6, K^bD^b) and its F_1 progeny (b x k, b x d, b x a) failed to mount a detectable response to H-$2K^b$ plus virus. This defect was detected in responses to challenges by either type A or type B influenza viruses. In contrast, mice congenic with the defective C57BL strain which share H-$2K^b$, but possess H-$2D^d$, were found to lyse infected targets which were compatible at H-$2K^b$. Using H-2 recombinant congenic strains, we were able to demonstrate that the gene(s) which controlled this immune response map in the H-2 gene complex. Several interpretations of these findings are discussed.

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II. INTRODUCTION

Immune response (Ir) genes, inherited in a simple mendelian fashion, regulating the antibody responses of mice and guinea pigs to chemically defined antigens were described almost 20 years ago (1). Recently, genetic regulation has been observed to extend to the elicitation of cytolytic T lymphocyte (CTL) responses to chemically modified self antigens (2-4), to minor histocompatibility antigens (5,6) and to several viruses (7-11). Doherty and his coworkers (12) initially described the nonresponsiveness to H-2K^b plus influenza virus. In these studies, we have further explored the phenomenon of lack of response to H-2K^b plus virus, and present evidence that genes located near the H-2D locus regulate the CTL response to H-2K^b plus influenza type A or type B viruses.

III. METHODS

A. *Viruses*

Influenza viruses A/PR/8/34 (PR8 virus; HON1), A/Japan/308/57 (Japan virus; H2N2), A/X-31 (a recombinant between A/HK/8/68 and A/PR/8/34; H3N2; 13), and B/Lee/40 were originally obtained from Dr. Jerome L. Schulman (Mt. Sinai School of Medicine, New York). Viruses were propagated in 10-day embryonated SPF chicken eggs (Spafas, Norwich, CT) and stored in allantoic fluid at -80°C. β-propriolactone inactivated Sendai virus was purchased from Connaught Laboratories, Ltd. (Willowdale, Ontario, Canada) and used as previously described (14).

B. *Mice*

All mice were used between 8 and 14 weeks of age. Female BALB/c mice were obtained from Charles River Breeding Farms (Wilmington, MA). Female C57BL/6 (B6), C57BL/10 (B10), B10.D2, B10.BR, A/J, B10.A, AKR/J, (C57BL/6 x A/J)F_1 (B6 x A), and (C57BL/6 x DBA/2J)F_1 (B6 x D2) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Female C3H/StHa and (C3H x C57BL/6)F_1 (C3 x B6) mice were purchased from Health Research, Inc. (West Seneca, NY). In addition, B10.A(3R), B10.A(5R), B10.A(2R), B10.A(4R),

B10.A(6R), B10.GD, B10.G, B10.MBR, B10.D2(R106), (B6 x AKR)F_1, and C3H.OL mice were obtained from the Harvard Medical School breeding colony.

C. *Immunization*

Mice were inoculated parenterally with 100 HA units of virus. One to four weeks after priming, mice were sacrificed and their spleens used as a source of immune lymphocytes.

D. *Generation of Cytotoxic Effector Cells In Vitro*

Primed spleen cells (7×10^6) were cocultivated with 5×10^6 syngeneic 1500 R irradiated virus infected lymphocytes for five days in 16 mm diameter culture wells (Linbro, Hamden, CT) in 2 ml complete medium (RPMI-1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, and 5×10^{-5} M 2-mercaptoethanol). Cultures were also established with uninfected syngeneic stimulator cells or medium alone; these control cultures failed to yield antiviral effector cells. After five days culture at 37°C in a 5% CO_2 environment, effector cells were harvested, washed, counted, and assayed for specific cytolytic activity.

E. *Assay of Antiviral Cytolytic T Cells*

In many experiments, thioglycolate induced peritoneal exudate cells were used as targets; two days prior to assaying cultures, mice were inoculated with 1 ml sterile thioglycolate broth. On the morning of the assay, mice were sacrificed and their peritoneal cavities were washed with Hanks balanced salt solution (HBSS) supplemented with 1% fetal bovine serum, penicillin and streptomycin, 15 mM Hepes buffer, and 1% heparin-sulfate solution. In other experiments, P815 ($H\text{-}2^d$), EL-4 ($H\text{-}2^b$), and RDM-4 ($H\text{-}2^k$) tumor cells passaged in ascites were used as target cells. In all experiments, both virally infected (10 EID_{50} per cell) and uninfected cells were used. Targets were pulsed with 100 μCi $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) for 1 hr and then washed extensively to remove free label. Varying numbers of effector lymphocytes or normal spleen cells were combined with 10^4 target cells in a total volume of 0.2 ml; all determinations were done in duplicate. After 4 hrs of incubation at 37°C, chromium release was measured by counting an aliquot of the supernatant fluid and has been described in detail

(15). Percent specific release was calculated by the formula:

$$\% SR = 100 (E-C)/(F-C)$$

where E is ^{51}Cr release in the presence of immune lymphocytes, C is ^{51}Cr release in the presence of nonimmune lymphocytes, and F is total releasable counts as determined by four freeze-thaw cycles.

IV. RESULTS

A. *Failure of H-2^b and F$_1$ Hybrids to Respond to H-2K^b Plus Virus*

Our initial experiments confirmed the observation of Doherty and his coworkers (11). B6, C3H, and (B6 x C3H)F_1 mice were immunized with influenza A/PR/8/34 (PR8) virus (HON1), the primed splenic lymphocytes were challenged in vitro with syngeneic cells infected with PR8 virus. Cytolytic effector cells were assayed against ^{51}Cr labeled, thioglycolate-induced peritoneal exudate cells (PECs) infected with the same virus. The results, shown in Table 1, indicate that while virus specific B6 effector cells lysed both infected B6 (K^bD^b) and B10.A(2R) (K^kD^b) targets, they failed to lyse infected B10.A(5R) (K^bD^d) targets. No specific lysis was observed on infected H-2 incompatible B10.D2 (K^dD^d) or B10.BR (K^kD^k) targets. In contrast, CTL effector cells obtained from (C3H x B6)F_1 and C3H mice were able to recognize target cells expressing either H-2K^k or H-2D^k plus virus. Cells from the (C3H x B6)F_1, like the B6, did not lyse virus infected B10.A(5R) targets, indicating an inability to recognize H-2K^b plus virus. However, the F_1 effectors were able to recognize the infected B6 target, suggesting that H-2D^b could be a suitable target.

We also studied several other F_1 hybrids, including (AKR x B6)F_1 (H-2^k x H-2^b), (B6 x A)F_1 (H-2^b x H-2^a), and (B6 x DBA/2)F_1 (H-2^b x H-2^d). Virus immune F_1 effector cells effectively lysed H-2D^b plus virus, but failed to lyse targets sharing only H-2K^b plus virus (Table 1). These F_1 responses suggest that H-2 genes in the trans chromosome position were unable to influence the ability to CTL to recognize H-2K^b plus virus.

Table 1. Lack of Detectable H-2K^b Restricted CTL Response to Influenza A/PR/8/34 Virus

Exp	Influenza virus-specific effector cells[b]	Haplotype	Net specific response from influenza virus infected PEC targets[a] B6 K^bD^b	C3H.OL K^dD^k	B10.BR K^kD^k	B10.A(2R) K^kD^b	B10.A(5R) K^bD^d	B10.D2 K^dD^d	B10.A(6R) K^qD^d	B10.MBR K^bD^q
I	B6	K^bD^b	31[c]	0	10[d]	38	7[e]	0	nd[f]	nd
	C3H	K^kD^k	0	21	57	46	3	0	nd	nd
	(B6 x C3H)F_1	K^bD^b/K^kD^k	31	14	20	47	3	0	nd	nd
II	B6	K^bD^b	68[g]	nd	nd	58	8	nd	nd	nd
	AKR	K^kD^k	nd	nd	27	28	nd	nd	nd	nd
	(B6 x AKR)F_1	K^bD^b/K^kD^k	46	nd	47	57	9	nd	nd	nd
III	(B6 x A/J)F_1	K^bD^b/K^kD^d	34[h]	nd	nd	42	nd	21	18	5[i]
	(B6 x DBA/2)F_1	K^bD^b/K^dD^d	56	nd	nd	nd	nd	36	nd	9
	A/J	K^kD^d	2	nd	nd	54	nd	35	nd	0
	DBA/2	K^dD^d	0	nd	nd	nd	nd	38	nd	3
	B6	K^bD^b	55	nd	nd	nd	nd	0	nd	7

[a]Thioglycolate-induced peritoneal exudate cells (PECs) were infected with influenza A/PR/8/34 virus and used as targets in a 4 hr CTL assay. Release on uninfected targets was less than 10%. Spontaneous release was 10-35% in the 3 experiments shown below. [b]Mice were primed parenterally with 100 HAU of A/PR/8/34 virus 1-4 weeks prior to secondary stimulation in culture with the same virus; 5 days later, CTL activity was assayed at varying effector to target ratios. [c]E/T=20:1. [d]Release of less than 10% was considered to be insignificant. [e]In experiments I and II, BALB/c effector cells lysed infected B10.A(5R) targets 47% and 54%, respectively. [f]Not determined. [g]E/T=20:1. [h]E/T=20:1. [i]B10.G (K^qD^q) effector cells lysed infected B10.MBR targets 35% at 20:1.

B. *Specificity of influenza CTL Response*

In other experiments, we examined the response when primed lymphocytes were presented with stimulator cells infected with viruses of other influenza type A subtypes (H2N2 and H3N2) or assayed on target cells infected with another type A influenza virus. These heterotypic CTL responses (16) also failed to elicit cytolytic cells which recognize H-2K^b plus virus from B6 or (AKR x B6)F$_1$ mice (Table 2). In addition, no crossreactivity by influenza virus effector cells was observed on PEC target cells which had been treated with Sendai virus (data not shown).

The inability of H-2^b mice and of (H-2^b x H-2^d)F$_1$ mice to recognize influenza virus plus H-2K^b was found to extend to CTL responses to influenza B/Lee/40 virus (Table 2), thus indicating that defective recognition extends to another type of influenza virus infected cells.

C. *Cytolytic Responses of H-2 Recombinant Mice*

To study the effect of H-2 genes on the specificity of the influenza specific CTL response, recombinant strains which carried the H-2K^b allele were used as responders. In our investigation of two such H-2 recombinants, we observed that B10.A(3R) and B10.A(5R) (both K^{b}D^d) strains significantly lysed infected B10 PEC targets. Data from a series of experiments is shown in Table 3. In every experiment, 3R or 5R responder cells demonstrated specific recognition of H-2K^b plus virus; the killing was highly reproducible and significant.

V. DISCUSSION

Target recognition by antiviral cytolytic effector cells requires the expression of both viral antigen(s) and MHC antigen(s) (reviewed in 17). Genes linked to the H-2 complex can regulate the ability to mount a CTL response to chemically or virally modified self. The genes controlling these CTL responses have been localized to the H-2K, H-2I, and H-2D regions (8,9,18). The mechanism(s) by which these genes influence the specificity of the CTL response may vary. The genes which map in the H-2I region are probably expressed in the responding cell population(s), while the genes that map in the H-2K and H-2D regions may be expressed as defects in

Table 2. Heterotypic and Heterologous Influenza Virus Responses of C57BL/6 and F_1 Mice

			Influenza virus-specific immune release from infected PEC targets					
			B6		B10.BR		B10.A(2R)	B10.A(5R)
Exp	Responder	Haplotype	(K^bD^b)		(K^kD^k)		(K^kD^b)	(K^bD^d)
I	B6[a]	K^bD^b	73:68[b]		5:2		66:58	0:8
	(B6 x AKR)F_1	K^bD^b/K^kD^k	61:46		52:47		56:44	1:9
	AKR	K^kD^k	nd		46:27		39:28	nd[c]
	B10.A(4R)	K^kD^b	33:26		47:39		65:53	0:0
	BALB/c	K^dD^d	nd		nd		nd	47:41
			B10 (K^bD^b)		B10.D2 (K^dD^d)		B10.MBR (K^bD^q)	
			A/PR/8/34	B/Lee/40	A/PR/8/34	B/Lee/40	A/PR/8/34	B/Lee/40
II	B6 αA/PR/8/34[d]	K^bD^b	62:55[e]	0:0	7:0	0:0	10:7	0:0
	αB/Lee/40	K^bD^b	0:0	72:63	0:0	0:0	0:0	0:0
	(B6 x D2)F_2 αA/PR/8/34	K^bD^b/K^dD^d	71:56	0:0	55:36	0:0	13:9	0:0
	αB/Lee/40	K^bD^b/K^dD^d	0:0	67:43	0:0	54:45	0:0	0:0
	DBA/2 αPR8	K^dD^d	11:0	0:0	49:38	0:0	0:0	0:0

[a]Mice were primed parenterally with A/PR/8/34 virus 10 days prior to sacrifice. Splenic lymphocytes from primed mice were cocultured with heterotypic A/HK/8/68 virus infected 1500 R irradiated syngeneic cells; 5 days later, effector cells were assayed in a 4 hr ^{51}Cr release assay at effector to target ratios of 60 and 20:1. Spontaneous release varied from 18 to 40%. [b]Two days prior to assay, mice were inoculated with thioglycolate to induce a peritoneal exudate. On the day of assay, cells were harvested by peritoneal lavage and a portion were infected with A/HK/8/68 virus. [c]Not determined. [d]Mice were primed parenterally with either A/PR/8/34 or B/Lee/40 virus 10 days prior to sacrifice. Splenic lymphocytes from primed mice were cocultured with

(Continuation of footnotes to Table 2)

1500 R treated syngeneic cells infected with the homologous virus; 5 days later, effector cells were assayed at effector to target ratios of 100 or 20:1. Spontaneous release varied from 20 to 33%. [e]Peritoneal exudate cells were obtained as described above but either infected with A/PR/8/34 or B/Lee/40 virus or left as uninfected control targets.

Table 3. CTL Responses of B10.A(3R) and B10.A(5R) Mice to Influenza Virus

Exp	Influenza virus-specific effector cells[b]	Net % ^{51}Cr release from influenza virus-infected target PECs[a] B6 (K^bD^b)	B10.D2 (K^dD^d)	B10.A(3R) (K^bD^d)
I	B6 (K^bD^b)	86:80:52[c]	nd[d]	0:11:6[e]
	BALB/c (K^dD^d)	nd	100:91:69	47:39:24
	B10.A(5R) (K^bD^d)	22:9:2	91:92:66	52:63:45
II	B6 (K^bD^b)	86:56[f]	9:0	0:0
	BALB/c (K^dD^d)	10:0	55:42	38:13
	B10.A(3R) (K^bD^d)	47:26	35:28	55:46

[a]Thioglycolate-induced peritoneal exudate cells (PECs) were infected with influenza A/PR/8/34 virus and used as targets in a 4 hr CTL assay. Release on uninfected targets was less than 10%. Spontaneous release was 10 to 35% in the 2 experiments shown below.
[b]Mice were primed parenterally with 100 HAU of A/PR/8/34 virus 1-4 weeks prior to secondary stimulation in culture with the same virus; 5 days later, CTL activity was assayed at varying effector to target ratios.
[c]E/T=100:33:11.
[d]Not determined.
[e]Not significant.
[f]E/T=75:25.

the stimulator cell population (19). A physical interaction on cell surfaces between viral and MHC antigen(s) has also been described (20,21). Thus, the inability to respond to MHC plus virus may be ascribed to association defects on the surface of infected or transformed cells: viral antigen(s) preferentially associated with one MHC antigen and not the other. Alternatively, the specificity of these CTL responses may be attributed to some previously undescribed Ir gene(s) which map near the right hand side of the MHC (7,10,12,18). It is also possible that defects in helper cell recognition of MHC plus virus complex result in the absence of CTLs generated to that complex.

Doherty and his coworkers found that H-2^b mice were unable to kill influenza virus infected target cells sharing only the left end of the MHC (12). The present report confirms these observations and extends them to both influenza type A and type B viruses (Table 2). However, we have observed that B10.A(3R) and B10.A(5R) mice, which share the H-$2K^b$, I-A^b and I-B^b loci with C57BL/6J mice but differ at the right hand side of the MHC, can recognize targets expressing H-$2K^b$ plus influenza virus glycoproteins (Table 3). These findings suggest that gene(s) mapping to the I-E through H-2D regions of the MHC can regulate the ability to generate K^b plus virus CTL responses.

However, an important paradox exists in the data. (B6 x A)F_1 hybrid mice have the identical MHC products present in B10.A(3R) and B10.A(5R) animals (the F_1 hybrid mice also have an additional set of H-2 gene products), yet fail to lyse targets expressing H-$2K^b$ plus virus. One interpretation of this observation is that association of influenza virus antigens with H-$2D^b$ is much more efficient or stable than the association of viral antigens with H-$2K^b$. However, in the B10.A(3R) and B10.A(5R) strains, viral association can occur with both K^b and D^d. Thus, the D^d product may have a lower association rate with influenza virus gene product(s). Therefore, in the F_1 hybrids, similar competitive interactions of virus and MHC products may occur, resulting in a hierarchy of effective H-2 plus virus complexes.

If this hypothesis is correct, and data from other systems seem to add support to this concept (20,21), we must still explain why influenza virus can associate with K^b products in C57BL peritoneal exudate cells (comprised of macrophages and T cells) to serve as effective targets. This second anomaly may be explained if we assume that the requirements for induction of CTL are more stringent than the

requirements for the effector phase of the CTL response. There are some precedents that the requirements for induction and elicitation of delayed type hypersensitivity responses may also differ (22).

A third interpretation of the above data places the genetic control at the level of the T cell, as opposed to the previous hypothesis which assumes the genetic control is expressed at the level of the antigen presenting cell. This T cell defect might either be in the helper cell or in the cytolytic T cell precursor.

Thus, our data suggest either antigen presenting cell, CTL precursor, or helper T cell defect in H-2^b mice or a hierarchy of immunodominance to virus plus H-2 antigens. Gomard and her associates (10) described a similar situation in the response of H-2^b and B10.A(5R) mice to Moloney virus antigens. They concluded that there exists a hierarchy of H-2 plus virus antigen complexes which elicit the CTL response, and ruled out the possibility of defective antigen presentation by target cells. In contrast to the lack of response to H-$2D^d$ plus Moloney virus (10), H-$2D^d$ plus influenza virus is preferentially recognized by CTLs. These findings are entirely consistent with our own observations in the influenza virus system.

We are currently extending our observations with other H-2 recombinant strains and their F_1 progeny and are exploring the nature of the hierarchy of antigen complexes which initiate the CTL response to influenza virus antigens.

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REFERENCES

1. McDevitt, H.O., and Benacerraf, B. (1969). *Adv. Immunol. 11,* 31.
2. Shearer, G.M., Rehn, T.G., and Garbarino, C.A. (1975). *J. Exp. Med. 141,* 1348.
3. Billings, P., Burakoff, S.J., Dorf, M.E., and Benacerraf, B. (1978). *J. Exp. Med. 148,* 341.
4. Levy, R.B., and Shearer, G.M. (1980). *J. Exp. Med. 151,* 252.
5. Simpson, E., and Gordon, R.D. (1977). *Immunol. Rev. 35,* 9.
6. vonBoehmer, H., Fathman, G., and Haas, W. (1977). *Eur. J. Immunol. 7,* 443.
7. Zinkernagel, R.M., Althage, A., Cooper, S., Kreeb, G., Klein, P.A., Sefton, B., Flaherty, L., Stimpfling, J., Shreffler, D., and Klein, J. (1978). *J. Exp. Med. 148,* 592.
8. Pfizenmaier, K., Trinchieri, G., Solter, D., and Knowles, B.B. (1978). *Nature (London) 274,* 691.
9. Mullbacher, A., and Blanden, R.V. (1978). *Immunogenetics 7,* 551.
10. Gomard E., Henin, Y., Colombani, M.J., and Levy, J.P. (1980). *J. Exp. Med. 151,* 1468.
11. Rosenthal, K.L., and Zinkernagel, R.M. (1981). *J. Immunol. 128,* 446
12. Doherty, P.C., Biddison, W.E., Bennink, J.R., and Knowles, B.B. (1978). *J. Exp. Med. 148,* 534.
13. Laver, W.G., and Kilbourne, E.D. (1966). *Virol. 30,* 493.
14. Finberg, R.W., Mescher, M., and Burakoff, S.J. (1978). *J. Exp. Med. 148,* 1620.
15. Lemonnier, F., Burakoff, S.J., Germain, R.N., and Benacerraf, G. (1977). *Proc. Natl. Acad. Sci. USA 74,* 1229.
16. Effros, R.B., Doherty, P.C., Gerhard, W., and Bennink, J. (1977). *J. Exp. Med. 145,* 557.
17. Zinkernagel, R.M., and Doherty, P.C. (1979). *Adv. Immunol. 27,* 51.
18. Meruelo, D. (1979). *J. Exp. Med. 149,* 898.
19. Emerson, S.G., Murphy, D.B., and Cone, R.E. (1980). *J. Exp. Med. 152,* 783.
20. Bubbers, J.E., Chen, S., and Lilly, F. (1978). *J. Exp. Med. 147,* 340.
21. Senek, A., and Neauport-Sautes, C. (1979). *J. Immunol. 112,* 1461.
22. Weinberger, J.Z., Benacerraf, B., and Dorf, M.E. (1979). *J. Exp. Med. 150:* 1255.

CHARACTERIZATION OF AN INFLUENZA VIRUS-RESISTANT MDBK CELL VARIANT

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I. ABSTRACT

We have established an influenza virus-resistant cell line which was obtained from cultures of MDBK cells that were initially persistently infected with the WSN strain of influenza A and subsequently lost the ability to produce virus. These variant cells are now almost completely refractory to influenza virus infection but retain the ability to support the growth of several other viruses including VSV, Sindbis virus, adenovirus and herpes simplex virus. All evidence indicates that this resistant cell variant was in fact derived from the completely permissive MDBK line. In view of these properties, we have termed these cells MDBK/IV to denote both their origin and their resistance to influenza virus.

We have taken several approaches to investigate the biochemical block that these cells have established to specifically prevent the replication of influenza virus. To this end, we are investigating (i) influenza virus binding and biochemical properties of the plasma membrane, (ii) the production of influenza virus-specific positive and negative strand RNA, (iii) the ability of the cells to accurately and efficiently translate influenza virus specific mRNA, and (iv) the possibility that virus-specific information is integrated into the

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cell genome.

Our experiments showed that MDBK/IV cells bound 80% less influenza virus than did the parental line and that this difference could not fully account for the lack of virus production. We have also detected the synthesis of virus-specific polyadenylated positive strand RNA in these cells and are currently characterizing this RNA and investigating its ability to be translated *in vivo* and *in vitro*.

II. INTRODUCTION

Genetically determined properties of cells are clearly important factors in determining whether they will be permissive or nonpermissive to a given virus. Very little is presently known about the nature of these determinants or about the mechanisms by which they restrict or foster virus multiplication. The availability of virus-resistant cell variants that show very limited differences from the permissive parent cell would be useful in studying these determinants. In the course of investigating persistent infection of MDBK cells with influenza virus, we have obtained a cell line which appears to be such a variant. These influenza virus resistant cells are fully susceptible to infection by several other viruses and thus should be useful in studies pertaining to host functions which are specifically required for influenza virus replication. The process by which these cells were derived, some biological properties of the cells and some preliminary biochemical studies will be the subject of this communication.

III. RESULTS

The influenza virus resistant variant cell line that we are investigating was obtained from MDBK cultures infected with the WSN strain of Influenza A (HON1). As previously described, stocks of this virus from chick embryo fibroblasts produce plaques of two morphological types, designated F and C (ref. 1). When confluent cultures of MDBK cells were infected with the less cytopathogenic of these two, the F plaque type virus, the monolayers remained intact after 48 hours at 37°, although the culture medium contained 10^7 to 10^8 PFU per ml. When the culture medium was replaced and again harvested 48 hours later, a drastic reduction in virus yield was observed but most of the cells in the culture remained intact. These cells could be subcultured and after about three weeks, the monolayers were free of cytopathogenic effects (CPE). As

shown in Fig. 1, virus yields dropped below the level detectable by hemagglutination. On day forty-five plaque assays were performed and, as expected from previous work (1), only C plaque type virus was present, the F plaque type having been eliminated by interference. Titrations carried out at 33° and 39.5° showed that the virus had not changed in temperature sensitivity.

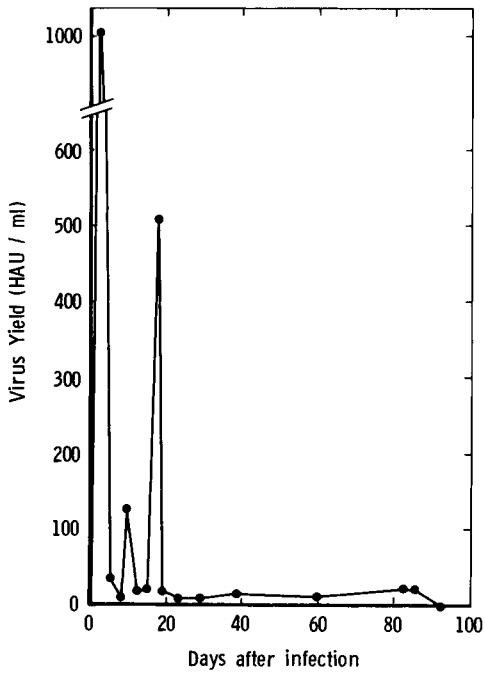

FIGURE 1. Influenza virus yields from persistently infected MDBK cells. Confluent monolayers of MDBK cells were infected with the F plaque type variant of the WSN strain of influenza A virus at a multiplicity of 0.005 PFU per cell as previously described (1). Culture medium was removed and replaced at 43 and 96 hours and at two to five day intervals thereafter. Cells were subcultured at 6 days and then at weekly intervals. Virus concentrations in the culture medium, measured as hemagglutinin units (HAU) per ml, were determined as previously described (1). From Schulze et al.; ref. 5.

When the C plaque type virus was used to initiate infection as above, the cultures went through the same course of events, except that extensive CPE was produced by 48 hours so that most of the cells were initially destroyed and more time was needed to obtain monolayers free of CPE.

Several different lines of cells were generated from different monolayers treated as above. Several of these lines were tested for their ability to form plaques when presented with a specific number of plaque forming units (PFUs) of vesicular stomatitis virus (VSV). These lines showed various plaquing efficiencies with VSV, but all were able to support the growth of the virus with at least 30% the efficiency of the parental MDBK cells. In contrast, two of the five lines tested were completely unable to support the growth of influenza virus (i.e., they produced no plaques when exposed to 10^5 PFU of influenza virus).

After about 100 days of culture, influenza virus was no longer detectable in the medium by hemagglutination or plaque assays independent of whether the cell cultures were originally infected with F or C plaque type virus. However, with the two completely resistant cell lines mentioned above, resistance to superinfection by influenza virus was retained even after virus production had completely ceased. Other more sensitive techniques aimed at detecting virus in these resistant cells also failed to show any virus production. These techniques included cocultivation of the resistant cells with susceptible MDBK cells and attempts to find a uridine-containing particle of density similar to that of influenza virions in the medium of the resistant cells.

The experiments described above indicated that persistently infected MDBK cell cultures could be established with either plaque type variant. However, some cultures regained susceptibility to influenza virus concomitant with spontaneous cessation of virus production while others retained complete resistance. Thus far, those which have remained resistant to superinfection after cessation of virus production were originally infected with the F plaque type virus, suggesting that a low level of cell destruction or concomitant growth of virus and cells during the initial stages of the infection may be involved in establishing an influenza virus resistant cell line.

We have chosen one of the resistant lines for further investigation. These cells appear to be stably resistant to influenza virus and have been grown in continuous culture for

about two and one-half years. Since they appear to be specifically resistant to influenza virus (see below), we consider them to be of great potential value for studying influenza virus replication and have conducted a variety of preliminary studies to test the feasibility of using the cells in this way. Our findings to date are summarized below.

A. Origin of the Influenza Virus Resistant Cells

In order to insure that the influenza virus resistant cells were indeed variants of MDBK cells rather than a contaminating cell, our current MDBK cell cultures (grown from cells originally obtained in 1969 from Dr. Purnell Choppin of the Rockefeller University) and line 13 were karyotyped in the laboratory of Dr. Patricia Monteleone in the Department of Pediatrics of St. Louis University School of Medicine. The modal number of chromosomes, determined from 100 cells of each type, was found to be 51 for each cell line. Statistical analysis of the chromosome distributions in the two cell populations indicated that neither distribution was normal, that the two populations were skewed in different directions with respect to chromosome number, but that the probability that the two were unrelated populations was small ($p = 0.09$). In addition, the chromosomes from the two lines were found to be virtually identical in morphology, both showing the same deviation from the karyotype expected from Bos taurus (cattle). Thus, the MDBK cells and the line 13 variant are apparently of common origin but their karyotypes provide evidence for low levels of divergence, as would be expected from two cell populations with different histories (in terms of exposure to virus) and independent cultivation over a period of two years.

Further support for the contention of a common origin of these two cell lines was obtained by restriction enzyme analysis of total cell DNA extracted from each line (Figure 2). The bands from highly repetitive DNA and/or satellite DNA, superimposed on the background smear of unique or nearly unique DNA sequences, was exactly the same for the two cell lines. This is as would be expected for the DNA of closely related cell lines and would not be observed if the variant line had actually been derived from an unrelated contaminant.

We have concluded that our virus resistant cells (line 13) are indeed MDBK cell variants and have designated them MDBK/IV (MDBK bar Influenza Virus) to denote both their origin and their resistance to influenza virus.

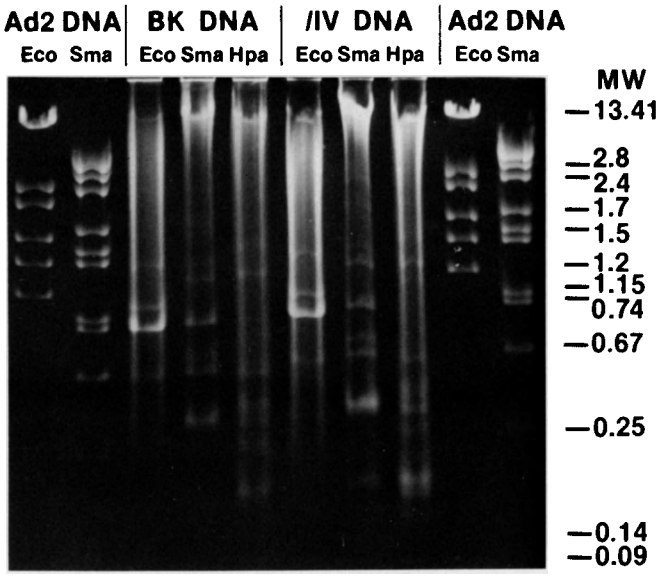

FIGURE 2. Restriction enzyme digestion of total cellular DNA from MDBK or MDBK/IV cells. Total DNA was extracted from cells by treatment with proteinase K + SDS followed by phenol extraction and ethanol precipitation. Two µg of adenovirus 2 DNA (lanes 1,2,9 and 10) or 10 µg of cell DNA (BK, lanes 3,4,5;/IV, lanes 6,7,8) were digested with the indicated restriction enzymes at 37° for 5 hrs then subjected to electrophoresis in a 1.4% agarose slab gel containing ethidium bromide. The gel was photographed under UV light.

B. Morphology and Growth Properties of MDBK/IV Cells

Although MDBK and MDBK/IV cells are indistinguishable in morphology when the cultures are sparse, extensive vacuolation occurs when MDBK/IV cultures are retained after they have reached confluency. However, the growth rate of the cells at low densities is not affected since the doubling time of both cell lines during logarithmic growth is approximately 15 hours under the conditions which we routinely use for cell growth.

C. Virus Susceptibility of MDBK/IV Cells

We have tested the susceptibility of MDBK/IV cells to

five different viruses in an attempt to determine to what extent the block in virus production is restricted to influenza virus replication. The data in Table 1 show that several other viruses can replicate efficiently in the variant cells. The numbers in parentheses represent the relative amounts of virus produced in the parental or variant cell line. The production of adenovirus was not quantitated.

TABLE 1. Susceptibility of MDBK and MDBK/IV Cells to Various Viruses

Virus	MDBK	MDBK/IV
Influenza	+	-
VSV	+	+ (2x)
Sindbis	+ (4x)	+
Adenovirus 2	+	+
HSV-1	+ (4x)	+

D. Attempts to Identify the Mechanism of Influenza Virus Resistance in MDBK/IV Cells

Our initial attempts to locate the resistance block in MDBK/IV cells were aimed at determining their efficiency of binding of influenza virus and thus investigate the possibility that these cells may have lost the receptor for influenza. When monolayers of the two cells were exposed to the same concentrations of labeled virus and the bound virus was measured as described (2) we found that the variant cells bound only about 20% as much virus as MDBK cells. This reduction in binding paralleled a reduction in glycosphingolipid content of the plasma membrane. The reduction in binding shown by these experiments was significant but was presumed to not fully account for the complete resistance of the variant cells to influenza virus. We therefore undertook a line of investigation to measure intracellular production of viral macromolecules as briefly described below.

Table 2 presents some preliminary data concerning the production of various species of viral RNA in the MDBK/IV cells. Following exposure to high multiplicities of influenza virus, polyadenylated cRNA was detected in these cells. Early in infection the amount of poly A(+) cRNA exceeded that found in MDBK cells, but did not increase in amount at about three hours after infection as it did in MDBK cells. In addition, we found that polyadenylated cRNA was the only viral RNA species which we could detect. Thus, late in infection when MDBK cells contained poly A(+) and poly A(-) cRNA as well as viral genome RNA, MDBK/IV cells contained only polyadenylated transcripts. These experiments suggested that MDBK/IV cells were fully capable of supporting primary transcription. Therefore, the cells could not have been blocked at penetration or uncoating of the virus; the transcription complex of the input virions had obviously been released into the cytoplasm of the cells and had been transcribed. We have so far been unable to detect the synthesis of poly A(-) RNA in the variant cell line.

TABLE 2. Influenza Virus Poly A(+) RNA in MDBK and MDBK/IV Cells[a]

Time after infection (hrs)	MDBK Cells		MDBK/IV Cells	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
1.75	150	225	450	2050
2.75	200	325	500	2025
3.75	725	8250	525	1625

[a]MDBK and /IV cells were infected with 30 PFU per cell and at the times indicated the nuclear and cytoplasmic fractions of the cells were isolated, the RNA was extracted from each and was fractionated into poly A(+) and poly A(-) by chromatography on oligo-dT cellulose. Each sample was then hybridized at 67° in 0.6 M NaCl to ^{125}I-vRNA, treated with pancreatic ribonuclease, precipitated with TCA and counted. The techniques and calculations used to determine virus genome copies were essentially those of Barrett *et al.* (3).

Failure to detect secondary transcription and complete cRNA transcripts to serve as templates for genome replication would be expected if MDBK/IV cells were unable to synthesize any viral proteins (4). Indeed our preliminary investigation of protein synthesis in these cells suggested that no viral proteins were made. By using indirect immunofluorescence and antisera prepared against the whole virus (i.e., mainly the NA, HA and M antigens) we have found the resistant cells to be essentially devoid of these major viral antigens. MDBK/IV cell cultures incubated for long periods (up to 30 hours) after exposure to 10 PFU per cell continued to show only occasional cells (1 to 2% of the population) which contained viral antigens. In contrast, these antigens were detectable in virtually all MDBK cells by this technique (5).

Experiments employing pulses of ^{35}S-methionine are currently underway to determine whether any of the viral proteins, including the nonstructural proteins and the P proteins, are present in the resistant cells under experimental conditions which show them to be present in MDBK cells. Thus far we have not detected any viral proteins in MDBK/IV cells (Figure 3) but we have not yet attempted to optimize conditions such as time and labeling period to observe the transient synthesis of the less abundant proteins. The number of primary transcripts present in these cells is certainly sufficient to provide the messenger RNA necessary for production of significant amounts of viral protein. Since we cannot detect viral protein synthesis, we tentatively conclude that the variant cells are blocked at the level of translation of viral RNA either because of defective RNA or by virtue of having generated translation factors that specifically exclude viral messengers.

IV. DISCUSSION

Based on the information which we have accumulated thus far, we consider these virus resistant cells to be very valuable for invesatigating influenza virus replication for the following reasons: (i) they are closely related genetically to an established cell line which is completely permissive for the strain of influenza virus which has been used extensively in the study of virus replication. (ii) These cells

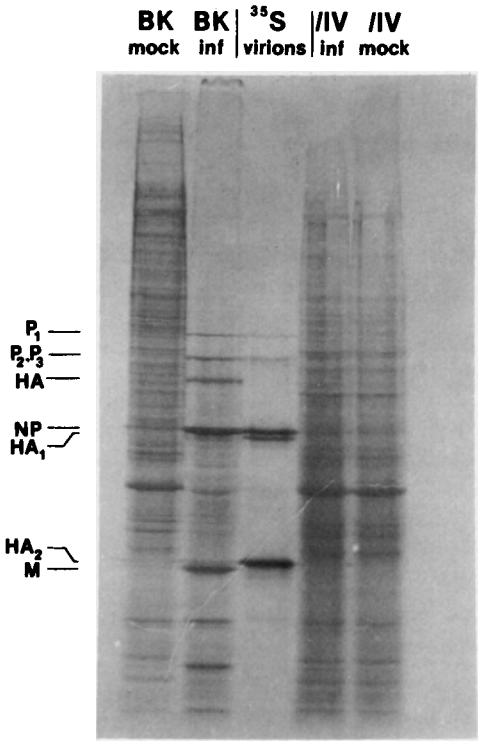

FIGURE 3. SDS polyacrylamide gel electrophoresis of infected and uninfected MDBK and MBDK/IV cells. MDBK and MBDK /IV cells were infected with apprximately 5 PFU/cell. Cells were labeled at 6 hr. post-infection with [^{35}S]methionine (100 µCi/ml) for 15 min and immediately harvested. Samples containing approximately 70,000 cpm (TCA precipitable) were separated on a 5-13% slab gel and protein bands were detected by autoradiography.

are specifically resistant to influenza virus suggesting that they differ from the parent cell in some property which is critical to influenza virus replication but not to other viruses. (iii) Our data indicate that the block is probably at some step subsequent to uncoating and prior to the synthesis of viral proteins, resulting in a situation in which only primary transcription can occur. If this proves to be the case, these cells constitute the only system in which this initial synthetic step of influenza virus replication can be studied in vivo without the use of inhibitors.

Aside from their use in the study of influenza virus replication, these cells provide an excellent opportunity to study mechanisms other than viral interference by which cells gain resistance to influenza virus. Our existing data have essentially eliminated interferon as the mechanism by which these cells have become resistant. Protection by interferon should prevent the growth of heterologous challenge viruses but, in fact, the variant cells are even more sensitive to VSV than are the parent cells and are also susceptible to several other viruses. Secondly, we have observed that mixed monolayers, obtained by growing MDBK cells and MDBK/IV cells together, produce turbid influenza virus plaques at 75% the efficiency of MDBK monolayers alone. If MDBK/IV cells were either constitutive or inducible high level producers of interferon they would protect the MDBK cells from infection and thereby prevent all plaque development. Protection of MDBK/IV cells by defective interfering particles would require that some infectious virus be available to provide the missing gene products, and no infectious virus has been detected in these cells for months. Thirdly, their inability to prevent plaque formation in mixed monolayers also argues that they are not making defective interfering particles.

There appear to be at least two possible mechanisms by which these influenza virus resistant cells could have arisen. First, they could be genetically stable cell variants which have been selected by survival during long term exposure to influenza virus. Such variants could presumably arise by a number of mechanisms including mutation(s) in unique structural genes, alterations in the control of gene expression, or by amplification of specific cellular genes that inhibit or prevent certain aspects of virus reproduction.

Secondly, MDBK/IV could have been produced by the insertion of viral genetic material into the cellular genome. If this were the case, there is no way at present to predict what kind of alteration in cell function would be observed.

Certainly, there is no a priori reason to expect that integration of influenza virus genetic information into a cell genome would render the recipient specifically resistant to infection with influenza virus.

ACKNOWLEDGMENTS

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REFERENCES

1. Noronha-Blob, L. and Schulze, I. T. (1976) Virology 69, 314.
2. Lakshmi, M. V. and Schulze, I. T. (1978) Virology 88, 314.
3. Barrett, T., Wolstenholme, A. J. and Mahy, B. W. J. (1979) Virology 98, 211.
4. Hay, A. J., Lomniczi, B., Bellamy, A. R. and Skehel, J. J. (1977) Virology 83, 337.
5. Schulze, I. T., Whitlow, K. J., Crecelius, D. M., and Lakshmi, M. V. (1981) in "Replication of Negative Strand Viruses", D.H.L. Bishop and R.W. Compans (eds.), Elsevier North Holland, Inc. (in press)

NEUTRALIZATION OF INFLUENZA VIRUS BY ANTIBODY: ATTACHMENT, UPTAKE AND UNCOATING OF NEUTRALIZED VIRUS IN CHICK EMBRYO CELLS

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ABSTRACT

We have investigated the attachment, uptake and uncoating by chick embryo fibroblast cell monolayers of an influenza type A virus neutralized to over 99.9% with heterogeneous rabbit antibody specific for the viral haemagglutinin. The rate of these processes was slightly slower than with non-neutralized virus but by 10 min. after inoculation there was no significant difference. We have thus demonstrated that neutralized virus is capable of undergoing a number of complex interactions with the cell with slight impairment of efficiency but not in extent. We consider that neutralization cannot be explained by an abrogation of the virus-cell receptor interaction and call into question the theory by which neutralizing antibody renders virus non-infectious.

I. INTRODUCTION

Neutralizing antibody is the main defence in higher vertebrates against reinfection with viruses (1). Its role in terminating primary infections is less clear but it is said to be important in limiting viraemia and in preventing expression of disease (2). However in recovery from certain virus infections (e.g. encephalomyocarditis virus (3), poliovirus (4), yellow fever virus (5)) circulating antibody is at least a contributory factor.

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It is axiomatic that the reaction between virus and neutralizing antibody leads to a loss of infectivity and this can be measured by titration of the virus in animals or cells in culture. The immunoglobulins (Ig) of classes G, M and A all have neutralizing activity (2). IgG is the most abundant, ubiquitous and widely studied but there are few data to explain how the combination of an infectious particle with neutralizing IgG leads to loss of infectivity. It is a widely held view (judging from textbooks of virology) that antibody interferes in the reaction between virus and its receptor on the cell surface. While this may be true with high (but probably non-physiological) concentrations of antibody, it is difficult to reconcile with data which show that neutralization follows first order kinetics and hence that one molecule of antibody per particle is sufficient to render it non-infectious (6,7). Even the counter arguments that neutralization is, after all, a multi-hit process (8,9) postulate that only a few molecules of antibody per particle are required for neutralization. A priori it is difficult to comprehend how these interfere with virus-receptor interactions when the surface structure of many viruses is composed of a multitude of receptor-binding molecules. Another line of argument shows that binding of antibody molecules to the surface of viruses does not necessarily impair infectivity (10-12) and may, in the presence of cells bearing receptors for antibody, even enhance infectivity (13-15).

Thus we felt it timely to discard our preconceptions and to approach the phenomenon of neutralization with an open mind and modern techniques. To this end the influenza virus system offers overwhelming advantages including considerable knowledge of the structure, antigenicity and functions of the components of the virus particle (16-18), two surface antigens the haemagglutinin (HA) and neuraminidase (NA) of which only antibody to the former is neutralizing (10), detailed information of the antigenicity of the HA through a battery of monoclonal antibodies (19-21) and the recent studies resulting from X-ray diffraction analysis of the molecular structure of the HA (22,23). There is also available much information about the molecular aspects of multiplication (16,17) and about the initial events of attachment, penetration and uncoating of influenza virus particles (24-28).

II. RESULTS

In the experiments described below we have used A/FP/Rostock/34 (H7N1) (35) grown in embryonated hen's eggs. ^{32}P-labelled virus was prepared (29) in monolayers of chick embryo fibroblast (CEF) cells (30) maintained in phosphate-free Eagle's medium and purified as previously described (29). Antibody to the HA of FP/R was made in New Zealand white rabbits by injecting on days 0 and 21 about 10^5 HAU of A/FP/Dutch/27 (H7N7) which has a closely related HA to FP/R and an unrelated NA. Serum was obtained by venous puncture on day 27. Antibodies to type-specific antigens were removed by repeated adsorption with ether-disrupted A/HK/1/68 (H3N2) (31) after which no antibody to the NP antigen could be detected. The antiserum had a haemagglutination-inhibiting titre against 4 HAU in the conventional test of 1/16000.

Radiolabelled virus ($10^{3.5}$ HAU; $10^{5.3}$ PFU:HA) was neutralized by incubation for 1h. at 25°C with antibody purified by affinity chromatography on protein A-Sepharose (Pharmacia Ltd). A portion was then removed to measure the decrease in infectivity by plaque assay on CEF cells, while the remainder was chilled and 100 µl inoculated onto CEF monolayers equilibrated to the temperature of an ice bath to ensure that only the initial stages of infection took place (24). A control virus preparation was similarly treated with an equivalent mass of antibody purified from pre-immunization rabbit serum and this caused no loss of infectivity. The amount of antibody used was defined operationally as that required to neutralize the infectivity of about 10^3 HAU by over 99.9%, in practice about 1 µg antibody per 10^7 particles.

Fig. 1 shows that the kinetics of association of non-neutralized and neutralized virus to CEF cell monolayers were similar. By 10 mins. after inoculation about 60% of the non-neutralized virus added to cells had attached compared with about 45% of neutralized virus, a difference of only 15%. After 30 mins. about the same amount of non-neutralized and neutralized virus had attached. Thus although there was a slight delay in the rate, the extent of attachment of neutralized virus to cells was unimpaired. Indeed over many experiments using different batches of virus, different radiolabels and different batches of cells, the amount of neutralized and non-neutralized virus which associated with cells by 60 mins. after inoculation was always very similar.

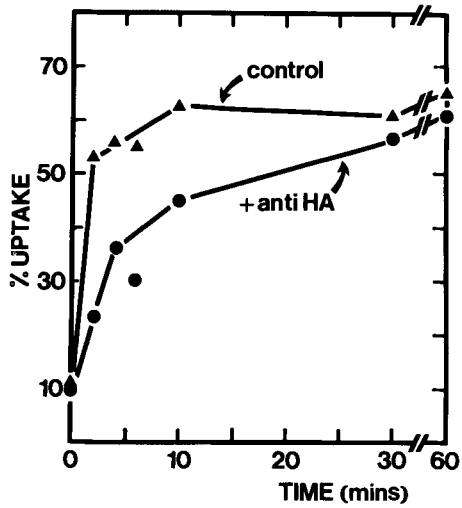

Fig. 1. Rates of uptake (i.e. association) of non-neutralized and neutralized ^{32}P-labelled FP/R by CEF cell monolayers. Virus was first incubated for 1h. at 25°C with purified anti-HA or with an equivalent mass of immunoglobulin purified from pre-immunization rabbit serum (control). The latter did not neutralize and antibody to HA resulted in a loss of 99.98% infectivity by plaque assay. In the figure virus-antibody mixtures were chilled and 100 µl inoculated onto monolayers in 50 mm Petri dishes held in an ice-bath. This represented a multiplicity of about 170 virus particles per cell. At the required times monolayers were rinsed thoroughly with cold phosphate buffered saline and scraped from the dish. The ordinate shows the amount of TCA-precipitable radioactivity associating with the cells as a percentage of that inoculated ($\sim 10^5$ cpm).

The second experiment measured the fate of virus attaching to cells in Fig. 1. Previous work demonstrated that soon after attachment virus is internalized by the cell (26) and then the RNA of the inoculated virus moves rapidly to the nucleus (24,25,32) while the viral envelope remains cytoplasmic (24,25). Nuclear-associated RNA can only be observed if cultures are inoculated at temperatures around 4°C since at above approximately 20°C the RNA moves from the nuclei back into the cytoplasm (24). Thus if monolayers inoculated as

described for Fig. 1 are fractionated into nuclei and cytoplasm the proportion of total cell-associated RNA in the nuclear fraction indicates the extent of uncoating (and ipso facto distinguishes between virus which has penetrated into the cell and that attached to the exterior surface). The estimate of uncoating will be a minimum value since we cannot distinguish between virus which is attached to the outside of the cell and that which is in the cytoplasm.

Fig. 2 shows that initially the majority of TCA-precipitable radioactivity was in the cytoplasm but by 10 mins. after inoculation the majority was nuclear. With non-neutralized virus nuclear localization was complete by 10 minutes.

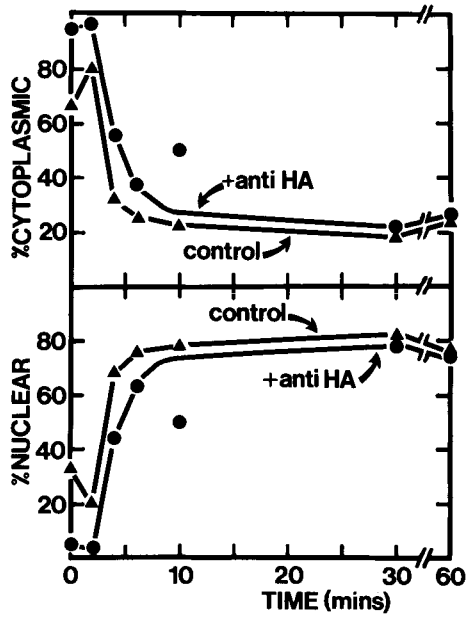

Fig. 2. Rate of uptake of non-neutralized and neutralized ^{32}P labelled FP/R into cytoplasmic and nuclear fractions of CEF cells. Virus was neutralized and inoculated as described in Fig. 1. At intervals monolayers were fractionated on ice by the nuclear monolayer method (23,24) using 2% Nonidet P40. Nuclei of comparable purity were obtained by Dounce homogenization also using NP40 detergent and these methods have been carefully characterized by a number of different criteria. The amounts of TCA-precipitable ^{32}P radiolabel were determined and plotted as a percentage of the combined total.

Neutralized virus moved more slowly and initially as much as 95% was cytoplasmic. However nuclear localization proceeded at approximately the same rate as with non-neutralized virus and interpolation shows that by 10 mins. the maximum amount of RNA from neutralized virus had become nuclear, antibody thus causing approximately a 2 min. delay. It seems likely that the delay here is due to internalization of neutralized virus or to its uncoating or some other stage prior to nuclear accumulation since this rate did not differ from the control. Note the high proportion (75 to 80%) of the RNA of cell-associated virus which became nuclear.

III. DISCUSSION

The data presented above show conclusively that virus neutralized by 99.98% attaches to, penetrates and uncoats in CEF cells to the same extent as non-neutralized virus. Kinetic studies show that the rate of attachment of neutralized virus, the rate of internalization and/or the rate of uncoating of neutralized virus is slightly less than that of non-neutralized virus but this difference is eliminated by 10 mins. after inoculation. The slower rate of attachment could conceivably be caused by neutralized virus experiencing difficulty in orienting with cellular receptors. We conclude that the mechanism of neutralization of influenza virus is not simple interference with the initial virus-cell receptor interactions. Further studies to elucidate how the virus becomes non-infectious are in progress (Possee, Schild and Dimmock, in preparation) and these show that the RNA of neutralized virions is not degraded and that no viral proteins are synthesized. However activity of the virion-associated transcriptase of neutralized virus was found to be impaired both *in vivo* and *in vitro*. We should also like to know the fate of antibody bound to virus after neutralized virus is internalized by the cell and the number of molecules of antibody bound per virus particle.

Finally we would point out to anyone wishing to reproduce these experiments that some batches of CEF cells do not take up influenza virus efficiently at 4°C. We routinely freeze the primary cell suspension while testing representative monolayers for their ability to internalize virus (26). Further to obtain a high proportion of nucleus-associated input virion RNA it is also necessary to use preparations of virus having a PFU:HA ratio of about 10^5 or greater.

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REFERENCES

1. Mims, C.A., in "The Pathogenesis of Infectious Disease" Chap. 9. Academic Press, New York, (1977).
2. Ogra, P.L., Morag, A. and Tiku, M.L., in "Viral Immunology and Immunopathology". (A.L. Notkins, ed.) p. 57. Academic Press, New York, (1975).
3. Murphy, B.R. and Glasgow, L.A., J. Exp. Med. 127, 1035 (1968).
4. Wyatt, H.V., J. Inf. Dis. 128, 802 (1973).
5. Zisman, B., Wheelock, E.F. and Allison, A.C., J. Immunol. 107, 236 (1971).
6. Dulbecco, R., Vogt, M., and Strickland, A.G.R., Virology 2, 162 (1956).
7. Mandel, B. Ann. N.Y. Acad. Sci. 83, 515 (1960).
8. Daniels, C.A., in "Viral Immunology and Immunopathology". (A.L. Notkins, ed.) p. 79. Academic Press, New York, (1975).
9. Della-Porta, A.J. and Westaway, E.G., J. Gen. Virol. 38, 1 (1977).
10. Webster, R.G. and Laver, W.G., J. Immunol. 99, 49 (1967).
11. Kjellen, L. and Pereira, H.G., J. Gen. Virol. 2, 177 (1968).
12. Lanni, F. and Lanni, Y.T., Coldspring Harb. Symp. 13, 159 (1953).
13. Hawkes, R.A. and Lafferty, K.J., Virology 33, 250 (1967).
14. Kilks, S.C. and Halstead, S.B., Nature (Lond.) 285, 504 (1980).
15. Peiris, J.S.M., Gordon, S., Unkeless, J.C. and Porterfield, J.S. Nature (Lond.) 289, 189 (1981).
16. British Medical Bulletin, 35, 1 (1979).
17. Tyrrell, D.A.J. and Pereira, H.G., Phil. Trans. Roy. Soc. B. 288, 287 (1980).
18. Laver, W.G. and Air, G., Structure and variation in influenza virus. Elsevier/North Holland, New York, (1980).
19. Gerhard, W., Topics in Inf. Dis. 3, 15 (1978).
20. Gerhard, W., Yewdell, J. and Frankel, M., in ref. 18, p. 273.
21. Gerhard, W. and Webster, R.G., J. Exp. Med. 148, 383 (1978).

22. Wilson, I.A., Skehel, J.J. and Wiley, D.C., Nature (Lond.) 289, 366 (1981).
23. Wiley, D.C., Wilson, I.A. and Skehel, J.J., Nature (Lond.) 289, 373 (1981).
24. Stephenson, J.R. and Dimmock, N.J., Virology 65, 77 (1975).
25. Hudson, J.B., Flawith, J. and Dimmock, N.J., Virology 86, 167 (1978).
26. Stephenson, J.R., Hudson, J.B. and Dimmock, N.J., Virology 86, 264 (1978).
27. Huang, R.T.C., Rott, R. and Klenk, H-D. Z. Naturforsch. 28c, 342 (1973).
28. Rott, R. in ref. 18, p. 201.
29. Crumpton, W.M., Dimmock, N.J. and Avery, R.J. Virology 90, 370 (1978).
30. Morser, M.J., Kennedy, S.I.T. and Burke, D.C., J. Gen. Virol. 29, 19 (1973).
31. Dimmock, N.J., Virology 39, 224 (1969).
32. Mark, G.E., Taylor, J.M., Broni, B. and Krug, R.M., J. Virol. 29, 744 (1979).
33. Tsai, R.L. and Green, H., Nature New Biol. (Lond.) 243, 168 (1973).
34. Hudson, J.B. and Dimmock, N.J., FEMS Microbiol. Letts. 1, 325 (1977).
35. W.H.O. Expert Group., Bull. Wld. Hlth. Org. 57, 227 (1979).

HUMAN ADAPTATION TO INFLUENZA VIRAL EVOLUTION

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INTRODUCTION

This meeting on "Genetic Variation Among Influenza Viruses" recognizes, by its very existence, the evolution of influenza viruses and their ceaseless adaptation to their human hosts. But, of course, evolution involves all species and as Dubos has reminded us, man adapts also (1). How well or how poorly he has adjusted to this protean virus is the subject of these remarks, as well as some consideration of strategy for the future. I shall make the case that with man, adaptation is not only physiological but cogitative. While the effects of physiologic adaptation are limited, adaptation resulting from thought, manipulation and intervention have resulted in both the eradication of smallpox and the potential for nuclear warfare.

The term "influenza," unless qualified, usually implies disease caused by influenza A viruses. It may clarify our thinking if we appreciate that epidemiologically influenza A is not one, but two diseases. For the most part the virus behaves like its cousins, influenza B and C viruses, as an obligate human parasite. Paradoxically, as an obligate parasite, despite, or perhaps because of, its apparent efficiency in spreading rapidly and widely within the population, its existence in any fixed antigenic form is tenuous so that disease continues, not because of persistence of the original virus, but due to the efficiency of its replacement by successive lineal descendents. As a discrete entity the virus is ephemeral, constantly changing by mutation or reassortment. Influenza B and C viruses are either less efficient or less conspicuous in achieving the same result.

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Indeed, the lesser frequency and degree of their antigenic variation may reflect a lesser requirement for such variation for their survival in a population in which immuno-susceptibles are better preserved.

Influenza A occurs also as what I would term a semi- or pseudo-zoonosis in which form, as a nonlineal descendent of immediately antecedent viruses, it is uniquely invasive as a major antigenic variant (if indeed it is proper to call it a variant under these circumstances). Quite rarely, the disease may represent a true zoonosis, as seems to be the case with sporadic infection of man with swine viruses, but a growing body of molecular evidence supports the idea that major or pandemic variants are recombinants in which novel antigens are derived from animal sources in combination with genes from human viruses that facilitate passage through the species barrier (2-5). Strategies for the containment of the two kinds of influenza may differ, as I shall discuss later.

Physiologic (Non-cogitative) Adaptation to Influenza Viruses

Through the years human beings have evolved mechanisms of defense against all virus diseases, including influenza, but an immune system designed for response to conventional, i.e., fixed antigens, appears to have difficulty in response to sequential bombardment with related but different antigenic variants, as is the case with influenza. The price of original antigenic sin is maximal response to earlier, rather than contemporary, antigens. The complexity of specific immunologic defenses are only now being unraveled as we better understand cell mediated immunity and its partial dependence on the major histocompatibility complex.

As yet there is no convincing evidence of genetic variation in human susceptibility in this apparently universal disease. Nor would we expect that a disease with little lethal impact prior to, or during the reproductive period, would have had selective effects on the population with respect to resistance to its lethal potential, especially because such effects are multifactorial, reflecting abnormalities of host physiology and secondary bacterial invasion, as well as intrinsic viral virulence. While man has had opportunity to adapt to a "generic" respiratory pathogen called influenza virus, so that he is able to confine it nicely with a three day fever in most instances, such adaptation may not be adequate to deal with influenza variants as yet unborn. Thus, the possibility of a more virulent

virus arises not only from the mutability of the virus, but from the difficulties of the host in adaptation to a constantly changing entity.

Although man has adapted in his capacity to survive and flourish in the face of continued epidemics of influenza, his adaptation has been insufficient for him to escape enormous morbidity, significant mortality and great economic loss. Appreciating this, he has begun cognitive and manipulative approaches to the control of influenza.

Cogitative Adaptation to Influenza Virus

Cogitative adaptation to influenza viruses began with the first recovery of virus from man and its propagation in the laboratory (Table I). This event initiated a period which I shall term Phase I, which involved descriptive virology, the establishment of laboratory reservoirs of virus (seed stocks), and the isolation of mutants and demonstration of genetic recombination or reassortment. In chronologically successive but overlapping phases, Phase II was marked by beginning manipulation of the virus and attempts at disease prevention, and Phase III represented an increased liability of ecologic change as attempted intervention increased.

TABLE I. Human Adaptation to Influenza Viruses

I.	Non-cogitative "Physiological" Adaptation
	A. Genetic (?)
	B. Immunological-changing immunophenotype
II.	Cogitative Adaptation
	A. Phase I -(descriptive and basic virology)
	B. Phase II -(basic virology (cont.) and limited intervention)
	C. Phase III-expanded intervention (live virus vaccines, cloned genes, addition to genetic reservoir)

In phase II basic studies of viral structure, genetics and replication were inextricably linked to limited attempts at disease prevention with vaccines and amantadine. This

period saw no significant impact on the containment or the epidemiology of the disease, but witnessed the production and global distribution of recombinants as immunogens and laboratory reagents. In an ecologic sense, this represented the expansion of a new virus reservoir in nature, i.e., in the laboratory.

The third phase differs significantly from preceding human adaptations in 1) the use of live virus vaccines possessing induced mutations or reassorted genes, and 2) the cloning of influenza virus genes in E. coli. In this phase, man-made viruses have now breached the laboratory walls as they are deliberately introduced into the human population. With the recent evidence that recombination between human influenza viruses can occur in man in nature (6,7), we must assume that whether or not vaccine viruses are demonstrably contagious, their genes are subject to rescue and propagation by wild type viruses. Thus, they represent an addition to the total gene pool available for sampling by influenza viruses adapting to us. Accordingly, with a reassortant live virus vaccine, transmissibility or contagiousness must be redefined to acknowledge the possibility of the rescue, in whole or in part, of its genes.

The implications of distributing the genes of domesticated viruses are unpredictable and the script could take any turn. Should reassortment of vaccine and wild type viruses yield a highly contagious, non-disease producing virus, the problem of influenza might vanish overnight. This improbable result is not an impossible one because virulence and transmissibility can be dichotomous (8,9).

On the other hand, genetic roulette could also produce a virus of enhanced virulence if, for example, a new antigenic variant struggling for survival sampled some tried and true non-antigenic, non-attenuating gene from a "successful" human virus now represented in a vaccine.

It is, of course, extremely unlikely that those viral genes reposing in E. coli constitute any threat or significant expansion of the ecologic pool. Nevertheless, they exist and can be expressed and we must note their presence.

Future Strategies for the Control of Influenza (See Table II)

Having already hinted at the possibility of control of influenza through inadvertent ecological meddling, it seems appropriate to see whether modern technology offers new approaches to influenza control. In analyzing the present situation, it will come as no surprise that a rational

approach demands more answers than we now have and therefore requires a return to Phase I of cogitative adaptation to the virus.

Recognizing the existence of two epidemiologic types of influenza, we must learn much more concerning their interdependence. Is the disappearance of a subtype dependent upon, or independent of, the introduction of a new pandemic virus? If it is not simply a matter of the exhaustion of susceptibles in the population, then what determines the composition of the replacing virus? (H1N1 has not yet displaced H3N2, but its reintroduction in 1977 was not truly pandemic. Although globally distributed, the virus was principally restricted to younger members of the population.) Was H2N2 supplanted in 11 years by H3N2 virus because insufficient susceptibles remained or because the transmissibility of H3N2 was overriding?

Are there limits to interspecific reassortment as a mechanism for the generation of new human subtypes? What are the host constraints on acceptable, i.e., cleavable, hemagglutinin antigens, if any? Can avian viruses infect man? If the reservoir of subtype antigens capable of replication in man is more limited than we presently suppose, then perhaps even eradication of human influenza is not an impossible objective.

TABLE II. Future Strategies for Influenza Control

A. Eradication by "Pulsed" Mass Immunization

Premises:
1. Effective vaccines available for use in "pulsed" immunization
2. Origin of pandemic viruses depends on reassortment of animal and human viruses
3. Significant antigenic drift requires multistep mutation
4. No viral latency or persistence

B. Eradication by Control of Animal Reservoirs

Premises:
1. Animals are sources of major variants
2. Domestic animals are principal source
3. Virus cannot survive by sequential mutation alone

C. Limited Control by Immunization

Premises:

1. Common antigenic determinant(s) exist
2. Common antigen(s) can induce effective immunity
3. Infection-induced immunity can be achieved by:
   a) further modified live virus vaccines
   b) sequential immunization with neuraminidase and partially attenuated live virus

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Is Eradication of Human Influenza Feasible? At first glance influenza would seem to be a most unlikely candidate for eradication because of 1) antigenic variability and the apparently ceaseless supplantation of one strain by another, and 2) the existence of possible animal reservoirs. On the other hand, for the most part, in its interpandemic or usual form the virus behaves as an obligate human pathogen with a rather tenuous hold on life (at least in any stable antigenic form) and there is no evidence for persistence or latency of the virus in man. Also, if pandemic "shift" variants must be recombinant, they might remain irretrievably locked in their animal hosts if the opportunity for reassortment with human viruses is precluded.

Therefore, assuming the validity of the foregoing assumptions, mass or saturation immunization in pulses at fixed points in time might sufficiently reduce the load of circulating human viruses so that the opportunity for significant antigenic mutation was reduced below that required for virus survival. In this theoretical model, several successive rounds of immunization, each targeted early on the latest variant, might ultimately eliminate the virus.

However, if the entrance of new major shift variants into the population is favored by the absence of territorial competition, and does not depend upon their rescue with human viruses, then pandemic cycles might occur at even shorter intervals.

All this assumes 1) effective and acceptable vaccines and/or chemoprophylactic agents, and 2) that epidemiologically significant progression of antigenic variation involves multiple mutations in the hemagglutinan (an assumption for which there is published support [10-13]), and 3) economic feasibility.

Although theoretically defensible, the prospect of

implementing this bold approach to influenza seems remote. If so, then a secondary position could address the animal reservoirs as the apparent source of novel major antigenic variants. There is abundant precedent for the control of human diseases transmitted from animals by attacking them at the source. If domestic animals are the principal reservoir of new antigens, then selective immunization, or even slaughter, are not unreasonable approaches to the problem. If, as Laver and Webster have suggested, wild birds are epidemiologically important reservoirs (14) then this approach has obvious flaws. It is an urgent and critical matter, in my view, to determine whether recombinant or wild type viruses bearing avian antigens (other than those newly categorized as H3 or H7) (15) can infect man. If not, the prospect for ultimate control seems somewhat brighter.

More Conservative Alternatives. Accepting the unlikelihood of either mass immunization or control of viral reservoirs in the near future, what approaches remain? As a new approach, chemoprophylaxis may require development of less toxic substances than amantadine. However, it is probable that this defense, like immunoprophylaxis, soon will be compromised by viral mutational adaptations.

If, as some evidence suggests, there is a single antigenic determinant common to all influenza A viruses (16,17), then hyperimmunization with such an antigen, either derived or synthetic, conceivably might confound the problem of strain-specific antigenic variation involving other antigenic sites. It is likely, however, that this approach will require the use of adjuvants which in themselves may introduce new problems of human toxicity. Much evidence (reviewed recently by Stuart-Harris [18]) suggests the superiority of infection-induced immunity. However, the use of live virus vaccines introduces not only the ecological problems discussed earlier, but continuing problems of reversion to virulence on the one hand, and overattenuation, particularly with respect to subjects with pre-existing antibody, on the other. In view of these considerations, the strategy which I have proposed of a two stage immunization with a neuraminidase specific (inactivated) vaccine followed by infection with wild or partially attenuated virus appears increasingly attractive (19).

One thing is clear; influenza will never be controlled or even significantly affected by the continuation of present control measures at their present level.

REFERENCES

1. Dubos, R., "Man Adapting." Yale University Press, New Haven, (1965).
2. Rasmussen, A. F., Jr., in "Newcastle Disease Virus" (R. P. Hanson, ed.), p. 313. University of Wisconsin Press, Madison, (1964).
3. Kilbourne, E. D., Science 160, 74 (1968).
4. Webster, R. G., Campbell, C. H., and Granoff, A., Virology 44, 317 (1971).
5. Scholtissek, C., Rohde, W., VonHoynigen, V., and Rott, R., Virology 87, 13 (1978).
6. Young, J. F., and Palese, P., Proc. Natl. Acad. Sci., U.S.A. 76, 6547 (1979).
7. Bean, W. J., Jr., Cox, N. J., and Kendal, A. P., Nature 284, 638 (1980).
8. Schulman, J. L., and Kilbourne, E. D., J. Exp. Med. 118, 257 (1963).
9. Schulman, J. L., in "Aerobiology" (I. H. Silver, ed.), p. 248. Academic Press, New York, (1970).
10. Gerhard, W., and Webster, R. G., J. Exp. Med. 148, 383 (1978).
11. Laver, W. G., Air, G. M., Dopheide, T. A., and Ward, C. W., Nature 283, 454 (1980).
12. Verhoeyen, M., Fang, R., Min Jou, W., Devos, R., Huylebroeck, D., Saman, E., and Fiers, W., Nature 286, 771 (1971).
13. Gething, M. J., Bye, J., Skehel, J., and Waterfield, M., Nature 287, 301 (1980).
14. Webster, R. G., and Laver, W. G., in "The Influenza Viruses and Influenza" (E. D. Kilbourne, ed.), p. 308. Academic Press, New York, (1975).
15. Assaad, F. A., et al., Bull. Wld. Hlth. Org. 58 (4), 585 (1980).
16. Laver, W. G., Downie, J. D., and Webster, R. G., Virology 59, 230 (1974).
17. Virelizier, J. L., Postlethwaite, R., Schild, G. C., and Allison, A. C., J. Exp. Med. 140, 1559 (1974).
18. Stuart-Harris, C., J. Inf. Dis. 142, 784 (1980).
19. Kilbourne, E. D., J. Inf. Dis. 134, 384 (1976).

NATURAL AND UNNATURAL VARIATION IN INFLUENZA A(H1N1) VIRUSES SINCE 1977

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ABSTRACT

In 1977 influenza A(H1N1) virus reappeared into a unique environment; influenza A(H3N2) strains were continuing to circulate, and the majority of the population over the age of 25 years possessed naturally acquired immunity to virus with antigens of H1N1 subtype. The evolution of the virus in this environment has been followed by antigenic,

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molecular and biological analysis. Observations made include: (1) rapid development of naturally circulating antigenic variants detectable with heterogenous animal sera; (2) more frequent variation of one hemagglutinin epitope (264) than of others detected by testing natural strains with monoclonal antibodies; (3) laboratory confirmation of higher mutability of epitope 264 than other epitopes and 'unnatural' selection of significant antigenic variants with mutation in epitope 264, paralleling natural events; (4) demonstration of mixed infections of individuals with H1N1 and H3N2 strains; (5) identification of recombinant H1N1 viruses among natural isolates and cocirculation of these viruses with 'true' H1N1 strains and (6) increased or decreased virulence of 'naturally' and 'unnaturally' selected H3N2/H1N1 recombinant viruses. These findings with influenza A(H1N1) virus illustrate the diversity in properties of influenza virus that can rapidly develop by natural or 'unnatural' selection.

INTRODUCTION

The unpredictability of influenza virus was confirmed in 1977 by the remarkable appearance of influenza A(H1N1) strains at the same time as epidemics of influenza A(H3N2) virus were occurring. As reported through the World Health Organization (WHO) influenza surveillance system, the first isolates of the H1N1 virus were recovered in China during May 1977 and by November the virus was simultaneously causing outbreaks in Asian regions of the Soviet Union and in Hong Kong (1). In a few months the virus spread throughout the world and, as shown in Figure 1, appeared in the midst of an ongoing influenza A(H3N2) epidemic in the US (2).

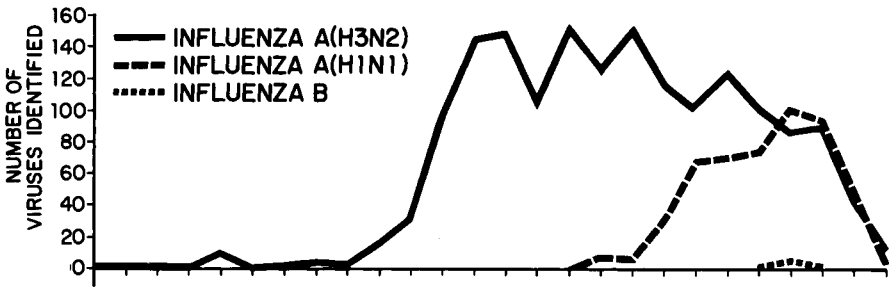

Fig. 1. Influenza isolations in the U.S. 1977-1978

The environment for the virus was totally unique. Firstly, the vast majority of the population over the age of about 25 years possessed naturally acquired immunity to virus with H1N1 antigens because of prior infections in previous periods of H1N1 virus prevalence, [which would now be recognized as extending from 1918 to 1956 (3)]. Thus, although the virus had available a large pool of totally susceptible persons under the age of about 25 years, a barrier of immunity existed that appeared to contain the virus within that population group (2). Secondly, the prevalent influenza A virus of the H3N2 subtype did not disappear but has continued to circulate simultaneously with influenza A virus of H1N1 subtype up to this time.

To follow the evolution of H1N1 viruses in this environment both traditional and modern methods have been applied from the time of the virus' reappearance in 1977. The antigenic and genetic similarity of the 1977 H1N1 virus strains to A/FW/1/50 which is representative of 'Scandinavian' strains from 1950-51 has been well documented (4,5,6). Also previously reported are the results of antigenic analysis of isolates from the 1977-1978 winter, which demonstrated the rapid appearance of several distinguishable antigenic variants. These variants were originally detected by their reduced inhibition with A/USSR ferret sera (2). They were also shown to have changed epitopes that could be recognized by a small panel of monoclonal antibodies (7).

Antibody prevalence surveys showed that 50% or more of the susceptible population of young persons were infected during the first two years' epidemics of H1N1 virus (8). Therefore, it seemed likely that additional antigenic variants should appear if the virus was to survive, and the propensity for antigenic variation by the H1N1 virus has been examined in detail by analyzing new natural isolates with post-infection sera from ferrets as well as with the monoclonal antibodies. In addition, we have studied 'unnatural variation' by using monoclonal antibodies to determine the frequencies with which mutation occurs within different epitopes when virus is passaged in laboratory hosts.

Another aspect of the evolution of the H1N1 virus studied since 1977 has been variation of its genome. During the period of cocirculation of H1N1 and H3N2 viruses in the United States at least one case of mixed infection by the two subtypes was demonstrated (9). Reports of similar events were received from investigators in the United Kingdom and Japan. This information facilitated the identification of viruses having recombinant genomes derived

from H1N1 and H3N2 strains, as found in the epidemic in the United States during the 1978-1979 winter (10). Although the recombinant virus had the hemagglutinin of one of the previously recognized antigenic variants, A/Brazil/11/78, genomic analysis of the first A/Brazil/11/78-like isolates from the preceeding winter (1977-1978) showed them to have a non-recombinant ('true' H1N1) genome. Thus, recombination and antigenic variation of the H1N1 viruses were independent events (11).

Because the recombinant H1N1 viruses were predominant in the U.S. in 1978-1979, and also became predominant in Japan (12), it appeared possible that they had an epidemiologic advantage over the 'true' H1N1 virus. We have therefore investigated the biological properties of the 'true' and 'recombinant' H1N1 viruses in human volunteer studies, and undertaken a prospective study of the genome composition of H1N1 influenza viruses isolated as part of the WHO influenza surveillance program.

RESULTS

Antigenic analysis of naturally occurring influenza A(H1N1) isolates.

There is no direct correlation between reaction patterns with monoclonal antibodies and with heterogeneous animal sera. In many cases viruses that have identical monoclonal reaction patterns are quite different when studied with post-infection ferret sera (Table 1).

Table 1. HI reaction patterns of natural influenza A(H1N1) variants that are differentiated by ferret sera but not with available monoclonal antibodies.

	Ferret sera			A/USSR/90/77 monoclonal antibody					
Antigen	A/USSR/92/77	A/Lackland/3/78	A/Texas/23/79	W18	22	70	110	264	385
A/USSR/90/77	640	1280	1280	3200	6400	12800	1600	6400	1600
A/Lackland/3/78	160	5120	320	<100	12800	12800	3200	<100	3200
A/Texas/23/79	40	640	640	<100	12800	12800	1600	<100	3200

On the other hand, monoclonal antibodies are often capable of detecting variation between viruses which cannot be distinguished from each other with ferret antisera to reference strains (Table 2).

Table 2. HI reaction patterns of natural influenza A(H1N1) variants that are differentiated by monoclonal antibodies but not by ferret sera to the variants.

Antigen	Ferret sera			A/USSR/90/77 monoclonal antibody					
	A/USSR/92/77	A/Brazil/11/78	A/England/333/80	W18	22	70	110	264	385
A/USSR/90/77	320	160	320	3200	6400	25600	1600	3200	400
A/Brazil/11/78	80	320	320	1600	3200	25600	1600	<100	400
A/England/333/80	40	160	640	1600	3200	6400	<100	<100	<100

Approximately 18 natural antigenic variants from A/USSR/90/77 have been detected that have unique reaction patterns when tested with ferret sera and monoclonal antibodies.

One noteworthy feature of the natural variants is that the vast majority of them have lost the ability to react with monoclonal antibody 264 (Fig. 2).

Change in the epitope recognized by this antibody was detected in variants identified in the 1977-1978 winter and one of these variants, A/Brazil/11/78, ultimately became the dominant H1N1 strain in many regions of the world. It is certainly possible, therefore, that this variant was the progenitor of many of the others and that this is the cause for the failure of so many natural variants to react with antibody 264. Alternatively the variety of natural variants with this changed epitope may have involved the occurrence of different mutational events in prototype, A/USSR/77-like, strains. We have approached this question by analysis of 'unnatural variants,' artificially selected in the laboratory.

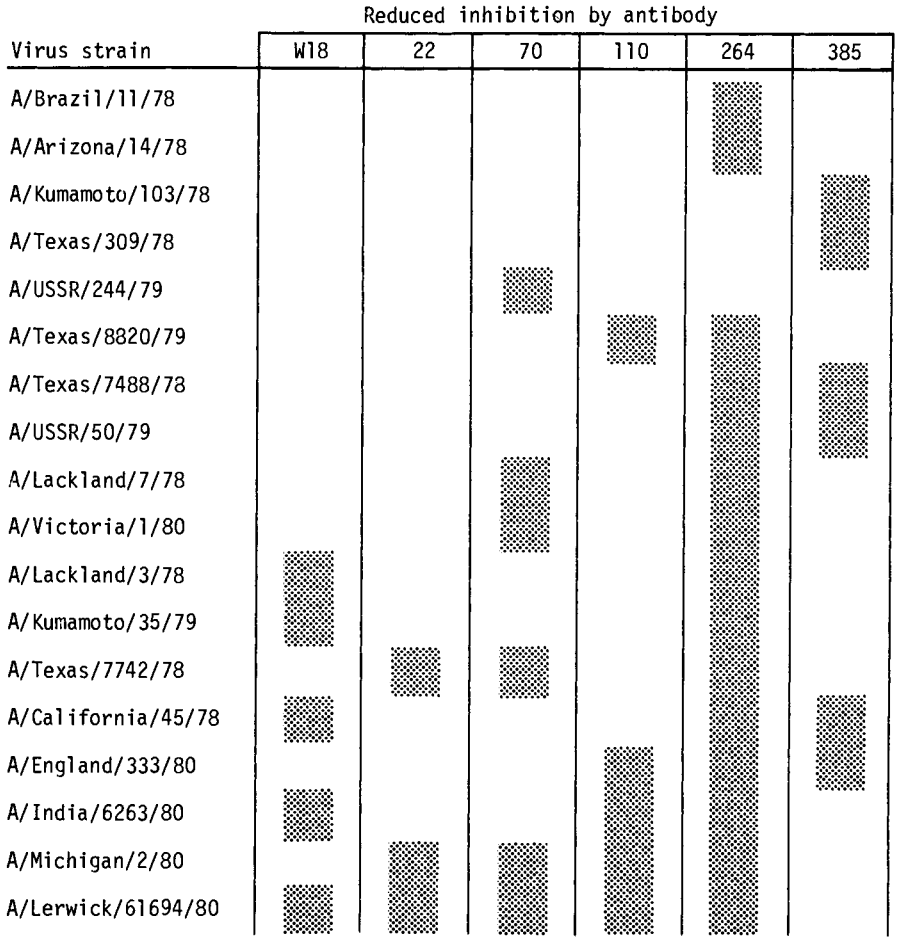

Figure 2. Monoclonal antibody reaction patterns of natural influenza A(H1N1) variants since 1977.

Prevalence of variants in cloned A/USSR/90/77 virus stocks.

Using procedures similar to those previously described by Gerhard, Webster and collaborators (13,14) to determine the frequencies with which epitopes vary in influenza hemagglutinins, we have compared the ability of six available monoclonal antibodies to artificially select H1N1 variants from A/USSR/90/77 virus. Cloned stocks of the virus were prepared by two plaque purification steps, and then neutralized with each monoclonal antibody. Virus

escaping neutralization was identified and quantitated by plaque titration. Prevalence of variants detected ranged from about 10^{-4} to $>10^{-7}$ according to the monoclonal antibody used for neutralization (Table 3).

Table 3. Prevalence of variants detected in cloned populations of A/USSR/90/77(H1N1) virus by breakthrough neutralization with monoclonal antibodies.

Monoclonal antibody used for selection	Immunoglobulin class	Prevalence of variants (log_{10}) in Clone 3-1	Clone 4-5
W18/1	IgG	-5.3	-5.1
22/1	IgM	-7.3	<-7.3
70/1	IgG	-5.1	-5.6
110/1	IgG	-5.2	-5.4
264/7	IgG	-4.2	-3.7
385/1	IgG	-5.3	<-5.3

The highest prevalence of variants was detected with the antibody 264, which parallels the situation observed with natural variants. The lowest frequency of variation was detected with antibody 22 which we know to be of IgM subclass. It is likely that the low frequency of escape of variants from antibody 22 relates to the extremely high avidity and multivalency of the IgM class antibodies. The frequency with which the variants were selected certainly corresponds to that previously shown with A/PR/8/34 and A/Memphis/1/71 viruses to be caused by point mutations (14,15).

Two other findings stem from this study of 'unnatural' variation of A/USSR/90/77 (H1N1) virus. Firstly, when the reaction patterns of variants, each selected with a single monoclonal antibody, were examined it was found that several of the monoclonal antibodies probably reacted with sites that interact with each other (Table 4). Thus selection of variants with antibody 22 always was associated with variation in the epitope recognized by antibody 70, and vice-versa. Selection with antibody W18 resulted in variants that had lost reactivity with antibody 264 but the reciprocal relationship was not observed. Change in the

Table 4. Grouping of artificially selected H1N1 variants according to their HI reaction patterns with monoclonal antibodies.

Group	Monoclonal antibody used for selection	HI activity of monoclonal antibody with antigenic variant					
		W18	22	70	110	264	385
A-1	70	+	-[1]	-	+	+	+
B-1	264	+	+	+	+	-	+
C-1	W18	-	+	+	+	-	-
C-2	W18	-	+	+	+	-	+
D-1	110	+	+	+	-	+	+
E-1	385	+	+	+	+	-	-
E-2	385	+	+	+	+	+	-
F-1	22	+	-	-	+	+	-

[1]- indicates HI titer ≥16-fold lower than that obtained with A/USSR/90/77 cl. 3-1 virus.

epitope recognized by antibody 385 occurred variably in these experiments and change in the epitope recognized by antibody 110 appeared to be independent of all other changes. These results therefore provide evidence for the existence of at least three distinct antigenic determinants on the A/USSR/90/77 hemagglutinin, one recognized by antibodies 22 and 70, a second by antibodies W18 and 264 and the third by antibody 110.

Secondly, when the variants artificially selected with individual monoclonal antibodies were analyzed with ferret antisera, several of them exhibited significant antigenic drift from the parental A/USSR/90/77 virus. In every variant where a change in the epitope recognized by antibody 264 had occurred significant antigenic drift from A/USSR/90/77 was evident, whereas in those variants which lacked a change in the epitope recognized by antibody 264 no antigenic drift detectable by ferret sera was evident even though other epitopes had changed (Table 5).

Table 5. Comparison of clones belonging to different groups of artificially selected variants for their extent of antigenic drift detectable with heterogenous ferret sera.

Group	Monoclonal antibody used for selection	Changed epitope	HI titer with three A/USSR/90/77 ferret antiserum: Overall mean	Fold change from homologous
Parent	-	-	640	-
A-1	70	22, 70	706	+1.1
B-1	264	264	107	-6.0
C-1	W18	W18, 264, 385	26	-25.0
C-2	W18	W18, 264	70	-9.0
D-1	110	110	508	-1.3
E-1	385	264, 385	160	-4.0
E-2	385	385	320	-2.0
F-1	22	22, 70, 385	1920	+3.0

This might be a reason why so many of the natural variants detected had lost the ability to react with antibody 264, because the primary reagents used in screening isolates were ferret antisera. The most important conclusion from the observations is that a point mutation in a critical locus of the H1 hemagglutinin, presumably in the determinant recognized by antibodies 264 and W18, could cause a degree of antigenic drift which might be epidemiologically significant. The degree of antigenic drift in some of the 'unnatural variants' was remarkable in that up to 25-fold lower reactivity with A/USSR ferret serum was demonstrated for variants of group C1, which were indistinguishable from a naturally isolated variant, A/Lackland/3/78, when tested with ferret sera (Table 6).

Genome Analysis of Influenza A(H1N1) Virus

We have used RNA-RNA hybridization analysis and competitive hybridization of individual genome segments (11), in addition to oligonucleotide mapping (10) of total vRNA to compare isolates with 'true' H1N1 strain such as A/USSR/90/77 and A/Brazil/11/78, and strains such as

Table 6. Comparison of HI reactions between natural variant A/Lackland/3/78 and 'unnatural variant' C-1 clone 1.

Antigen	Changed epitope	Ferret sera		
		A/USSR/90/77	A/Lackland/3/78	C-1 (-1)
A/USSR/90/77	-	640	640	120
A/Lackland/3/78	W18, 264	80	2560	320
In vitro variant C-1 (-1)	W18, 264, 385	80	3840	640

A/California/10/78 or A/California/45/78 which are known to be recombinants containing several genes derived from H3N2 virus (10,12). Our studies of viruses isolated from different regions of the world during 1978 to 1980 showed that recombinant H1N1 viruses were circulating in many countries (16). Young and Palese (17) recently demonstrated that some European isolates had an additional gene (RNA 7) of H3N2 origin. This result, together with results of antigenic analysis, enabled us to conclude that at least two recombinational events between H1N1 and H3N2 strains must have occurred (16). We also found that at the same time as these recombinant strains were circulating in 1979, 'true' H1N1 viruses were also still in evidence in some countries. 'True' H1N1 viruses caused sporadic illnesses and local outbreaks in the U.S. in February, 1980 at the same time as recombinant H1N1 strains were causing sporadic illnesses (16). Our most recent results of genomic analysis have shown that recombinant H1N1 strains were isolated on several occasions in 1980, including Singapore (January), Brazil (April/May) and Puerto Rico (September). These viruses were usually antigenically similar to A/Brazil/11/78 or A/USSR/90/77. Resurgence in several countries of influenza A(H1N1) in widespread cases, outbreaks and epidemics during 1980, however, was associated with new minor antigenic variants, represented by A/England/333/80 and related strains. These variants all failed to react with at least monoclonal antibodies 110 and 264 (Fig. 2, Table 2). Such variants were first isolated during April-July in England and India, and by early 1981 had appeared in North America, and many European countries, although A/Brazil/11/78 strains were still active in some places. Representative isolates of these antigenic variants from England, India, and the U.S. were shown by RNA

oligonuclcotide mapping to be genetically very similar to 'true' H1N1 strains rather than to the recombinant H1N1 virus (Figure 3). This was confirmed for an isolate from India and the U.S. by competitive RNA hybridization (not shown).

Thus, the epidemiology of influenza A(H1N1) in the U.S. and several other countries has seen since 1977 the reappearance of a virus closely related to the 'Scandinavian' strains from 1950, then epidemics of H1N1 viruses with reassorted genomes derived by recombinations of H1N1 strains with H3N2 strains, and subsequently spread of virus with a non-recombinant H1N1 genome and which exhibits modest antigenic drift from the original 1977 strain (Table 7).

Table 7. Detection in the U.S. of influenza A(H1N1) virus with different antigenic and genomic compositions Jan 1978-Jan 1981.

Period	HA	Recombinant[a] genome	Prevalence
May 1977-June 1978	A/USSR/90/77	-	Epidemic
	A/Brazil/11/78	-	Sporadic
July 1978-June 1979	A/USSR/90/77	+	Sporadic
	A/Brazil/11/78	+	Epidemic[b]
July 1979-June 1980	A/Brazil/11/78	+	Sporadic
	A/Brazil/11/78	-	Outbreaks
July 1980-June 1981	A/England/333/80	-	Outbreaks

[a]At least polymerase and nucleoprotein genes derived from influenza A (H3N2) virus.

[b]Associated with increased frequency of Reye syndrome cases.

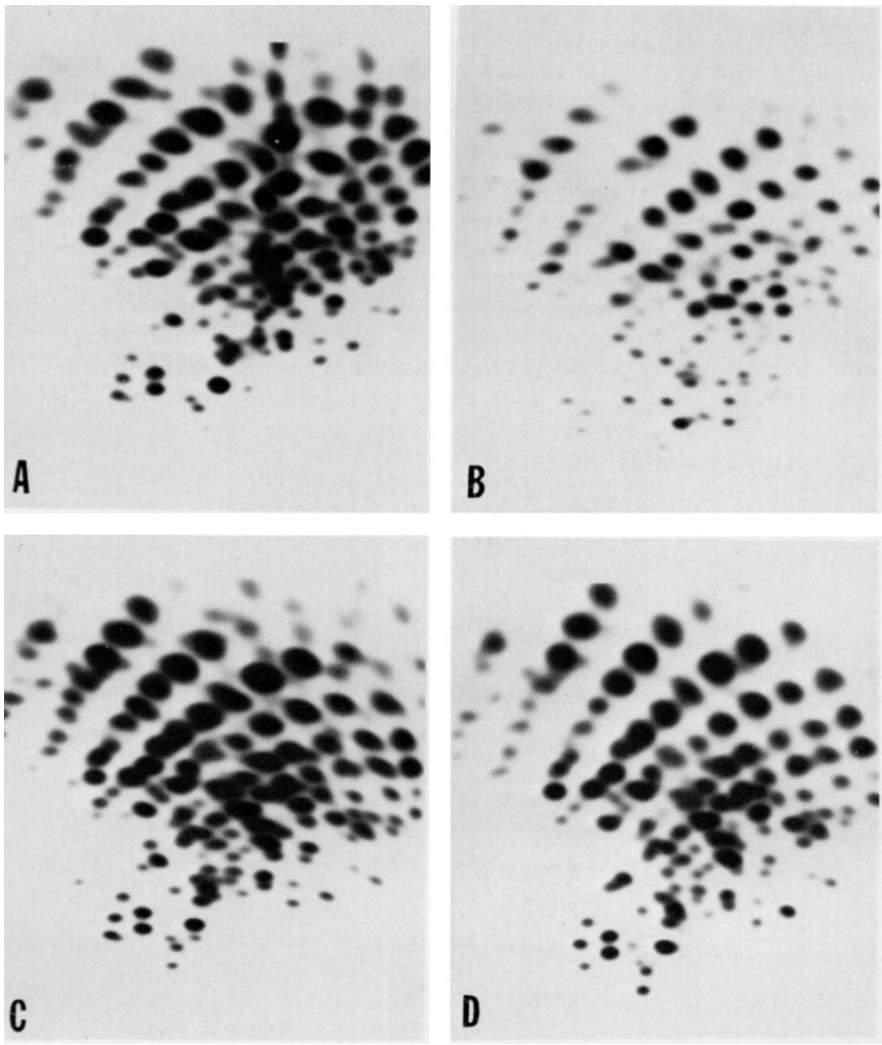

Fig. 3. RNA oligonucleotide maps of total virion RNA from influenza A(H1N1) isolates. (A) 'true' H1N1 virus A/Brazil/11/78; (b) recombinant H1N1 virus A/California/45/78; (C) A/India/6333/80 and (D) A/Memphis/7/80.

Human Volunteer Studies with Influenza A(H1N1) X Influenza A(H3N2) Recombinants

Isolation from mixed infections of recombinant H1N1 X H3N2 viruses in 1977-1978 (9), and the widespread occurrence in epidemic form during 1978-1979 of H1N1 recombinant viruses (10,11,12,16), stimulated us to evaluate in adult human volunteers the biological effect of recombination between human influenza viruses of two different subtypes.

Viruses studied included 'unnatural' recombinants containing H3 hemagglutinin, but one or more genes from H1N1 virus and which were cloned from mixed infections (18), 'true' H1N1 viruses from the first outbreaks following this virus' reappearance, and several natural recombinant H1N1 viruses isolated in the winter of 1978-1979.

The results, summarized in Table 8, show a general trend from which the following conclusions may be provisionally drawn. Firstly, several viruses containing H3 hemagglutinin but one or more genes of H1N1 virus origin had appreciably lower virulence for human volunteers than has previously been demonstrated for H3-containing isolates. This observation held true even when these viruses were given to human volunteers at the very high doses of up to $10^{8.5}$ EID_{50}. The findings, therefore, support the view that in this case mixing of genes from H3N2 and H1N1 viruses resulted in viruses having a virulence lower than that which would normally be expected for viruses of H3 antigenic subtype.

Secondly, the human volunteer studies with 'true' H1N1 viruses from 1977-1978 suggested that these viruses had a somewhat lower virulence than wild-type viruses of H3N2 subtype, although higher than the viruses with H3 hemagglutinin but recombinant genomes. Thirdly, when naturally circulating recombinant H1N1 viruses from 1978-1979 were tested these strains usually caused influenza illness which was of a severity quite comparable to that found with previous wild-type H3N2 viruses. This observation has been consistently repeated with virus antigenically and genetically similar to the H1N1 recombinant strain which in the United States caused epidemics in 1978-1979 (additional results not shown). Thus, it is suggested that viruses having H1N1 antigens similar to A/Brazil/11/78, but four genes of H3N2 origin, had increased virulence compared to 'true' H1N1 strains. One may speculate that an additional alteration in the recombinant H1N1 strains associated with enhanced virulence compared to 'true' H1N1 strain was the ability to cause Reye syndrome. An increased incidence of this disease occurred

Table 8. Summary of responses of volunteers (with pre-existing HI antibody titer ≤24) after intranasal instillation of recombinant and wild-type human influenza A viruses.

Volunteer group and virus given	Dose	No. tested	Laboratory evidence of infection			Clinical reactions		
			No. with virus isolates	No. with HI antibody rise	Total no. infected	Moderate/ severe	Nil/ mild	Average score of reactions
Wild-type H3N2 virus 1968-1973	10^5-$10^{6.4}$	10	8	8	8 (80%)	8 (80%)	2 (20%)	49
Wild-type H1N1 virus[1] 1977-1978	10^6-10^7	7	7	6	7 (100%)	4 (57%)	3 (43%)	20
Wyoming recombinants with H3 hemagglutinin, 1977-1978[2]	$10^{6.5}$-$10^{8.5}$	19	12	15	16 (84%)	5 (26%)	14 (74%)	12
Wild-type recombinants[3] with H1 hemagglutinin[1], 1978-1979	-$10^{8.5}$	5	4	5	5 (100%)	4 (80%)	1 (20%)	49

[1]All results involving viruses with H1 hemagglutinin were from volunteers born after about 1955, and having pre-infection HI titers of <6 to the infecting virus.

[2]Clones recombinants from mixed infection of Wyoming schoolboy, having either H3N2 or H3N1 antigens, but one or more other genes derived from H1N1 virus.

[3]Viruses A/California/10/78 and A/Georgia/64/79 from epidemics in U. S. These viruses have polymerase and nucleoprotein genes of H3N2 origin and other genes of H1N1 origin.

in the U.S. only in 1978/1979 when the recombinant H1N1 strains were prevalent, and not in 1977/1978 when epidemics of 'true' H1N1 virus occurred (19). Other viral or epidemiological factors may have contributed to this observation, however.

DISCUSSION

The observations we have made with human influenza A(H1N1) viruses in the three years since their reappearance has established several fundamental points about the virus evolution. For example, the use of monoclonal antibodies has shown that variation is occurring continually in diverse sites within the virus hemagglutinin. Most of these changes probably are of no epidemiological significance because they either do not lead to a reduction in the overall level of inhibition of the virus by antibodies resulting from infection with the prototype strain, or do not result in a new strain achieving widespread distribution. However, studies of artificially selected variants demonstrated that probably even a single point mutation is capable of giving rise to variants that do show a degree of antigenic drift that could be epidemiologically significant.

The factors that favor spread of some antigenic variants (eg., A/Brazil/11/78 and A/England/333/80) are quite unclear and might be related to mutations distinct from antigenic change. These above-mentioned variants did not require acquisition of genes from H3N2 virus, however, in order to displace the original A/USSR/90/77-like strains, even though recombinant H1N1 viruses have appeared naturally and at least for a period were probably the predominant viruses in several countries. An additional observation has been that the pathogenicity of human influenza viruses appear to depend on gene constellations, as has been shown for animal models. The findings well illustrate the difficulty of predicting, rather than observing, the effect of variation in influenza viruses that can be detected by laboratory procedures..

References

1. World Health Organization. (1979). Wkly. Epidem. Rec. 54, 25.

2. Kendal, A. P., Joseph, J. M., Kobayashi, G., Nelson, D., Reyes, C. R., Ross, M.R., Sarandria, J. L., White, R., Woodall, D. F., Noble, G. R., and Dowdle, W. R. (1979). Am. J. Epidemiol. 110, 449.
3. World Health Organization. (1980). Bull. WHO 58, 585.
4. Kendal, A. P., Noble, G. R., Skehel, J. J., and Dowdle, W. R. (1978). Virology 89, 632.
5. Nakajima, K., Desselberger, U., and Palese, P. (1978). Nature 274, 234.
6. Scholtissek, C., von Hoynigen, V., and Rott, R. (1978). Virology 89, 613.
7. Webster, R. G., Kendal, A. P., and Gerhard, W. (1979). Virology 96, 258.
8. World Health Organization. (1980). Wkly. Epidem. Rec. 55, 73.
9. Kendal, A. P., Lee, D. T., Parish, H. S., Raines, D., Noble, G. R. and Dowdle, W. R. (1979). Am. J. Epidemiol. 111, 462.
10. Young, J. F., and Palese, P. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 6547.
11. Bean, W. J., Cox, N. J., and Kendal, A. P. (1980). Nature 284, 638.
12. Nakajima, S., Nakajima, K., Takeuchi, Y., and Sugiura, A. (1980). J. Inf. Dis. 142, 492.
13. Yewdell, J. W., Webster, R. G., and Gerhard, W. U. (1979). Nature 279, 246.
14. Laver, W. G., Air, G. M., Webster, R. G., Gerhard, W., Ward, C. W., and Dopheide, T. A. A. (1979). Virology 98, 226.
15. Laver, W. G., Gerhard, W., Webster, R. G., Frankel, M. E., and Air, G. M. (1979). Proc. Natl. Acad. Sci. U.S.A. 76, 1425.
16. Nakajima, S., Cox, N. J., and Kendal, A. P. (1981). Inf. Imm. in press.
17. Young, J. F., and Palese, P. (1981). in "Replication of Negative Strand Viruses," (D. H. L. Bishop and R. W. Compans, eds.), Elsevier-North Holland, N.Y., in press.
18. Beare, A. S., Kendal, A. P., Cox, N. J. and Scholtissek, C. (1980). Inf. Imm. 28, 753.
19. Halsey, N.A., Hurwitz, E. S., Meiklejohn, G., Todd, W. A., Edell, T., Todd, J. K., and McIntosh, K. (1980). J. Pediatrics 97, 535.

HETEROGENEITY OF INFLUENZA VIRUSES ISOLATED FROM THE HOUSTON COMMUNITY DURING DEFINED EPIDEMIC PERIODS[1]

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ABSTRACT. Epidemic influenza occurred in Houston during four consecutive winters. Analysis of isolates from each year indicated that the predominant type was A/Victoria (H3N2) in 1976, B/Hong Kong in 1977, A/Texas (H3N2) in 1978 and A/Brazil (H1N1) in 1979. During each of the three type A epidemics, relatively low frequencies of type B virus were isolated, and, during the type B epidemic type A viruses were isolated. A serological characterization of H3N2 viruses isolated during 1976-1978 indicated that the A/Victoria variant was present during 3 consecutive winters and the A/Texas variant during the latter two. Late in the H3N2 epidemic of 1978, 126 viruses of the H1N1 subtype were isolated. Most of the H1N1 strains isolated during 1978 were antigenically similar to A/USSR but 7% were identified as A/Brazil, the dominant virus of 1979. These data indicate that influenza viruses of different types, subtypes and variants within a

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a subtype can circulate simultaneously in the same community. Moreover, variants were often identified in the community a full year before they produced epidemic disease. When more sensitive techniques were employed, extensive antigenic microheterogeneity among A/Victoria (H3N2) and A/USSR (H1N1) strains was evident but, a pattern of antigenic drift toward the succeeding variants A/Texas and A/Brazil was not apparent.

INTRODUCTION

Continuous virologic surveillance of the Houston Community was initiated during 1974 and influenza virus infections in epidemics of varying intensities have been evident during each of seven successive years. Analysis of isolates from each year indicate that each epidemic was produced primarily by a single variant of type A or B virus. However, multiple variants of a type A subtype were frequently isolated during the same winter and a relatively low frequency of infection with viruses of a different type or subtype have occurred almost every year. In some instances, the pattern of their isolation appeared to forecast the succeeding epidemic; this observation has been termed the "herald wave" phenomenon (Glezen et al, 1980). This chapter reports a characterization of influenza isolates collected through four successive epidemics (1975-1979) that will serve to illustrate these observations. In addition, two retrospective attempts to demonstrate antigenic drift toward succeeding variants within a subtype will also be described.

MATERIAL AND METHODS

Virus specimens were collected through the community-wide surveillance program at the Influenza Research Center at Baylor College of Medicine, Houston, Texas. The primary care facilities that served as a representative sample of families from all socioeconomic groups, the criteria for obtaining a throat swab specimen, and procedures used for isolation and characterization of influenza viruses have been fully described in previous publications (Baxter et al, 1977, Glezen et al, 1978, Glezen et al, 1980, Six and Kasel, 1979).

RESULTS

Distribution of influenza virus isolates by type and subtype during different epidemics; the herald wave phenomenon.

Type A influenza viruses similar to A/Victoria/3/75 (H3N2) produced an intensive epidemic in Houston during the winter of 1975-76, Table 1. During the latter one-half of the epidemic, a low frequency of infections with type B virus was detected. A total of 34 type B viruses were recovered (3% of the total influenza isolates, the first few isolates of which were serotyped as B/Hong Kong/72-like. This "herald wave" of viral isolates proved to forecast the epidemic strain for the succeeding winter. A similar phenomenon occurred near the end of the B/Hong Kong/72 epidemic of 1976-

TABLE I. Distribution of Influenza Virus Isolates for Different Epidemics in Houston, Texas[a]

		Percent Isolates		
Year	Type Subtype	A		B
		H3N2	H1N1	
1975-76		97	-	3
1976-77		6.8	-	93
1977-78		84	15	0.7[b]
1978-79		-	100	0.4[c]

[a] Portions of the data were taken from Glezen et al, 1978 and Glezen et al, 1980.

[b] Six influenza type B virus were recovered over the entire epidemic season.

[c] A single influenza B virus was isolated

77 when 51 (6.8% of total influenza virus isolates) infections by type A influenza viruses were detected (Glezen et al, 1980). The latter viruses were found to be a mixture of two variants of the H3N2 subtype, A/Victoria/3/75 and A/Texas/1/77, and this combination of variants produced the epidemic of 1977-78. The third consecutive "herald wave" occurred during the latter portion of this epidemic when 126 (15% of the total influenza isolates) viruses of the H1N1 subtype appeared. Characterization of a few isolates from the first wave indicated that they were antigenically similar to A/USSR/90/77 whereas the majority of the strains isolated during the epidemic of 1978-79 were similar to A/Brazil/11 /78.

Antigenic analysis of the hemagglutinin proteins of A/Victoria/3/75-like strains.

To determine if isolates within an epidemic exhibited antigenic drift toward a succeeding variant, strains of A/Victoria/3/75-like viruses were tested by competition radioimmunoprecipitation (RIP) assays. Twelve were isolated during the winter of 1976, four from each portion (early, intermediate, and late) of the epidemic; three strains isolated during 1977 and three others isolated during 1978 were also analyzed.

Two types of changes in the antigenic determinants on the hemagglutinin (HA) subunit were observed. Results of the experiment shown in Figure 1 illustrates one of these types of antigenic variation. In this experiment, the prototype A/Victoria/3/75 strain and three strains that were isolated early, intermediate, and late during the 1976 epidemic were used to inhibit the binding of ^{125}I-Vic-HA to homologous antiserum. The slope of the competition curve obtained with each of the strains was significantly different from the prototype strain. At high concentrations, each strain inhibited the reaction by greater than 85%, with no indication of a plateau. This result suggests that all the antigenic determinants were present on the HA of each of the strains but that an alteration had occurred in a portion of the antigenic determinants. Nine strains exhibited this type of pattern in competition assays and the slopes ranged from 15% to 36% lower than that observed with the prototype HA. In the second type of antigenic variation, three strains yielded competition curves that exhibited plateaus (figure 2). The presence of the plateau indicates that a portion of the antibody population was directed against antigenic epitopes on

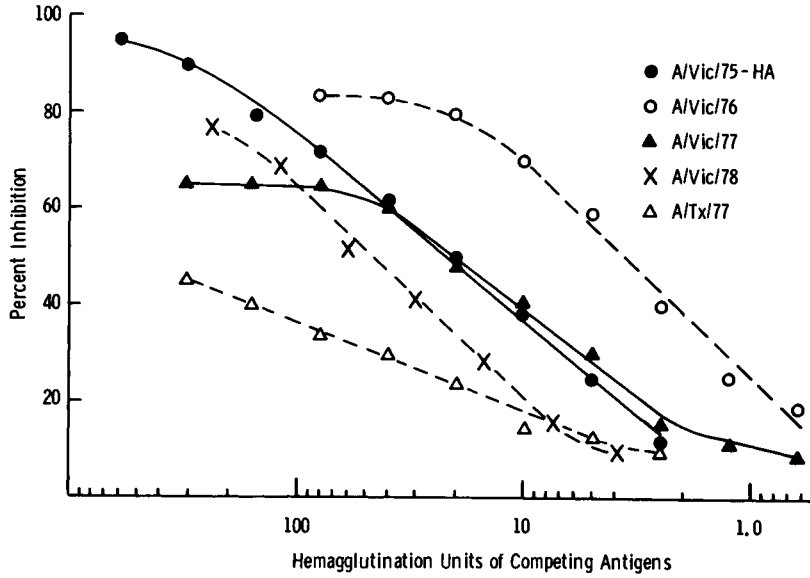

Figure 1. *Inhibition of* ^{125} *I-A/Vic/75-HA binding to homologous antibody by purified A/Vic/75-like viruses isolated during an epidemic.*

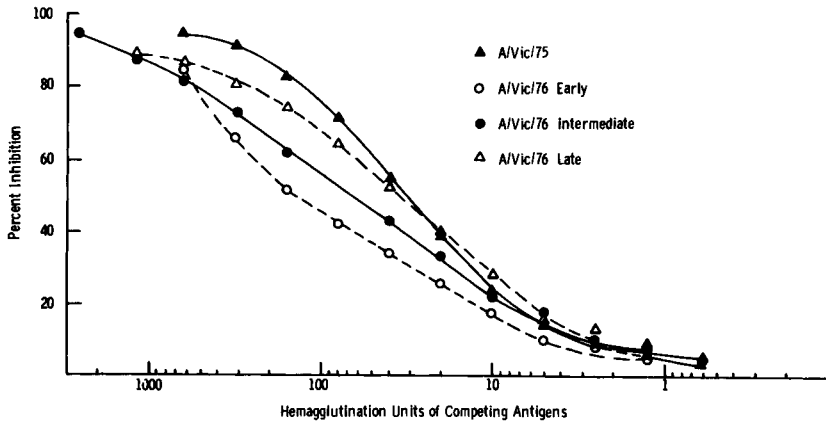

Figure 2. *Inhibition of* ^{125} *I-A/Vic/75-HA binding to homologous antibody by purified H3N2 viruses.*

the prototype A/Vic strain that were not recognized on the HAs of the competing strains. Strains exhibiting both type of reactions were isolated during all 3 winters (Table 2). Six of the 18 strains were indistinguishable from the prototype A/Victoria HA by this methodology.

For comparison, the antigenic relatedness of the A/Tex-HA to the A/Vic-HA is also presented in figure 2. The slope the A/Texas/1/77 competition curve is 50% lower than that obtained with the prototype A/Vic-HA and, although not shown here, the curve reached a plateau at approximately 65% inhibition with very high concentrations of competing antigen. This result indicates the existence of major differences in the antigenic determinants of the HAs of the two variants. Thus, the antigenic differences that were detected among the A/Victoria/3/75 isolates must reflect minor antigenic changes.

Table 2. Antigenic Heterogeneity Among A/Vic/75 Isolates Obtained in Different Epidemic Years

Year and Time of Isolation		Number of Strains: Tested	Not Identical To A/Vic/75[a]
1976[b]	Early	4	2
	Middle	4	2
	Late	4	3
1977		3	2
1978		3	3
TOTAL		18	12 (67%)

[a] Antigenicity of the HA proteins were assessed by competition RIP assays and strains that exhibited a plateau or 15% or greater reduction in the slope of the competition curve when compared to the A/Vic/75 (H3N2) prototype strains were considered to be different.

[b] The genetic composition of a portion of these 1976 strains was examined by oligonucleotide mapping and the gene coding for the HA protein was different for each (Palese et al, 1980).

Antigenic analysis of the HA proteins of H1N1 viruses isolated during 1978.

Since the "herald wave" of 1978 and the epidemic of 1979 appeared to represent infections by two different variants of the H1N1 subtype, specimens collected during 1978 were examined retrospectively for the presence of A/Brazil/11/78-like viruses. The virus isolates were evaluated by hemagglutination-inhibition tests using post infection ferret antisera to both variants and seven monoclonal antibodies prepared to the HA protein of A/USSR/90/77. The reaction patterns of these reagents with the reference strains are shown in Table 3. Ferret antisera to A/USSR/77 exhibited 4 fold lower titers with the Brazil strain, but antisera to latter variant was not as discriminating. The 264/2 and 264/7 monoclones were particularly useful in this study because these exhibited no crossreactivity with the A/Brazil/11/78 variant. While the five other monoclones reacted to comparable titers with both variants, previous studies have indicated that they recognize different antigenic epitopes on the HA molecule (Webster et al, 1980).

Table 3. Hemagglutination-Inhibition Reactions of Reference Influenza H1N1 Strains with Ferret Antisera and Monoclonal Antibodies

	Ferret Antisera	
	A/USSR/90/77	A/BRAZIL/11/78
A/USSR/90/77	160	160
A/BRAZIL/11/78	40	320

	Monoclonal Antibodies						
	264/2	264/7	385	W18	22	70	110
A/USSR/90/77	400	1600	800	1600	3200	12,800	800
A/BRAZIL/11/78	<200	<200	800	800	1600	6,400	800

A total of 126 H1N1 strains were isolated during 1978. Of 73 strains examined by HI tests, 54 exhibited titers comparable to the A/USSR/90/77 strain, 19 (26%) exhibited a four fold or greater reduction in their reactivity with one or more of the monoclonal antibody preparations and five were shown to be A/Brazil/11/78-like strains. While viruses that differed antigenically from A/USSR/90/77 were recovered throughout the epidemic period, the five Brazil strains were clustered in the last one third of isolates and in this respect, followed the pattern of previous "herald wave." In addition, isolates obtained from students involved in a different study during the latter one-third of the outbreak were also tested; 2 of 4 were antigenically similar to A/Brazil/11/78.

DISCUSSION

Influenza viruses regularly produce epidemic disease in human populations. Presently, the forecast of future epidemics is based on the serologic susceptibility of the general population to currently prevalent type A and B viruses and the degree of antigenic change in newly recognized variants. For early detection of novel variants, a world-wide surveillance system was established by the World Health Organization. In recent years, prospective identification of viruses collected through this system have illustrated the complexity of influenza epidemiology. Both type A and type B viruses are usually isolated during epidemic seasons; since 1977 recovery of type A viruses belonging to both the H3N2 and H1N1 subtypes has been a common finding. Antigenic variants of the prevalent epidemic strain are also usually recovered at a low frequency (Kendal et al, 1979; Periera, 1977; and Willers and Hopken, 1979).

Successive yearly, occurrences of influenza epidemics in Houston provided the opportunity to gain additional information on the epidemiology of influenza virus infections in a restricted geographic area.

The present data clearly demonstrate that viruses isolated from the same community during defined epidemic periods are antigenically heterogeneous. When large numbers of specimens were examined, a low frequency of infections by viruses of a different type or subtype than the epidemic strain was usually observed and, in three instances, the epidemiologic pattern suggested that succeeding epidemics were predictable. However, an epidemic by B/Singapore/79-like viruses was observed during 1980 and this virus was not recovered during the preceding winter. Thus the frequency with which

successive epidemics can be predicted by this phenomemon remains to be determined.

Analysis of H3N2 isolates by competition RIP tests, and H1N1 isolates by monoclonal antibodies revealed extensive microheterogeneity among strains recovered in the same epidemic year. Our failure to observe more dramatic antigenic changes among A/Victoria/75 (H3N2) strains, i.e., indicating a drift toward A/Texas/77 (H3N2), may be attributable to the limited number selected for examination. An evaluation of a relatively large number of H1N1 isolates recovered during the A/USSR/77 epidemic in 1977-78 using monoclonal antibodies resulted in the identification of variants. Retrospectively, five strains were typed as A/Brazil/78, the variant succeeding A/USSR/77. However, an evolutionary pattern of antigenic drift was not discernable. Variants shown subsequently to be more distantly related antigenically to A/USSR/77 than A/Brazil/78 preceded the isolation of the latter variant. This observation supports the conclusion that the epidemic potential of a strain does not appear to be based exclusively on its antigenic properties.

REFERENCES

Baxter, B.D., Couch, R.B., Greenberg, S.B., et al (1977). *J. Clin. Microbiol. 6*, 19.

Glezen, W.P. and Couch, R.B. (1978). *N. Engl. J. Med. 298*, 587.

Glezen, W.P., Couch, R.N., Taber, L.H. et al (1980). *Am. J. Epidemiology. 111*, 13.

Kendal, A.P., Joseph, J.M., Kobayaski, G. et al (1979). *Am. J. Epidemiol. 110*, 449.

Palese, P., Brand, C., Young, J.F. et al (1980). *In* "Perspectives in Virology" Vol. 11. Academic Press, New York.

Pereira, M.S. and Chakraverty, P. (1977). *J. Hyg. Camb. 79*, 77.

Six, H.R., and Kasel, J.A. (1979). *Virol. 96*, 588.

Webster, R.G., Kendal, A.P., and Gerhard, W. (1979). *Virol. 96*, 258.

Willers, H., and Höpken, W. (1979). *Med. Microbio. Immunol. 167*, 21.

BIOLOGIC AND GENETIC CHARACTERIZATION OF AN INFLUENZA A VIRUS ASSOCIATED WITH EPIZOOTIC PNEUMONIA IN SEALS

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I. ABSTRACT

An influenza A virus antigenically similar to A/FPV/Dutch/27 (Hav1Neq1) was isolated from harbor seals (Phoca vitulina) that had died of acute hemorrhagic pneumonia on Cape Cod Peninsula, USA, in the winter of 1979-1980. High titers of virus were obtained from the lungs and lower titers from the brains of the seals. Antigenic and genetic analyses of the seal isolates showed that all of the genes and gene products were closely related to different avian influenza viruses; however, biologically, the virus behaved more like a mammalian strain. The seal virus replicated and produced pneumonia in experimentally infected harbor seals, but the clinical course and pathology were less severe than in the natural infection. The virus also replicated in ferrets, cats and pigs but produced no disease. In avian species, the seal influenza virus replicated poorly, produced no disease signs and was not shed in the feces. During studies with experimentally and naturally infected seals, several individuals experienced conjunctivitis and high titers of seal virus were recovered from the eye of one individual, indicating that this virus can infect the human eye.

The A/Seal/Mass/1/80 influenza virus provides the first evidence suggesting that a strain deriving all of its genes from one or more avian influenza viruses can be associated

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with severe disease in a mammalian population in nature. Whether this breach of species specificity represents a unique event in influenza evolution remains to be determined, but raises the possibility that human or animal influenza viruses may be derived directly from avian strains.

II. INTRODUCTION

Influenza A viruses cause infection in a wide range of avian species but their natural host range in mammals is restricted to humans, pigs and horses (1). Influenza A viruses have been isolated periodically from dogs, cats, monkeys and cattle (1), but there is no evidence that the viruses are maintained in these species. Although there is considerable antigenic and genetic relatedness between the influenza A viruses of avian and mammalian species (2,3), there is no convincing evidence for infection of mammalian species with avian strains in nature.

In December, 1979, an unusually large number of stranded and dead harbor seals (Phoca vitulina) were found on the beaches of Cape Cod, Massachusetts, U.S.A. Peak mortality occurred in January, 1980, and postmortem examination of the seals revealed severe lung consolidation typical of viral pneumonia (4). The number of deaths has been estimated at 500 and represents at least a 20% mortality in the local seal population.

We now report the characterization of an influenza A virus from the lungs and brains of the dead harbor seals. The virus was antigenically similar to A/FPV/Dutch/27 (Hav1Neq1) (H7N7), but replicated preferentially in mammalian, rather than in avian, species.

III. RESULTS

A. Virus Isolation and Antigenic Characterization

Analysis of tissue samples from dead seals revealed high titers of influenza A virus in the lungs (10^6-10^7 EID_{50}/gm) and lower titers in the brains ($10^{1.5}$-$10^{2.5}$ EID_{50}/gm) (5,6). The hemagglutinin, neuraminidase, and nucleoprotein of the seal isolates were characterized in

serological tests with reference antisera. The seal isolates were classified as Hav1Neq1 (H7N7) in hemagglutination-inhibition and neuraminidase-inhibition assays (7) with antisera to the hemagglutinin of A/FPV/Rostock/34 and the neuraminidase of A/Eq/Pr/1/56. In ELISA assays with monoclonal antibodies to the nucleoprotein of A/WSN/33 (8), the seal virus gave a reactivity pattern identical to avian viruses. The multiple isolates from seals were antigenically indistinguishable so one isolate, designated A/Seal/Mass/1/80 (Seal/Mass), was selected for additional studies.

B. Experimental Infections of Seals

Since the seal viruses were isolated from dead seals with pneumonia, it was necessary to determine if the virus would replicate and cause disease in seals. Four seals inoculated intranasally with 10^8 EID_{50} of Seal/Mass shed virus for 7 to 10 days post-inoculation (p.i.) with peak virus titers of 10^7 EID_{50}/ml of nasal wash (Table 1). The seals developed a mild cough and a mucopurulent discharge from the eyes and nose. Seals killed from 7-10 days p.i. showed macroscopic and histological evidence of pneumonitis.

C. Conjunctivitis in Humans Exposed to Infected Seals

During postmortem studies on the stranded and dead seals, 4 individuals developed conjunctivitis within 2-3 days of known contamination of the eyes. Subsequently, during experimental studies on infection of seals, an animal sneezed in the face of one of the investigators. The person developed severe conjunctivitis of the right eye and high titers of virus (10^5 EID_{50}) were isolated from conjunctival swabs. The virus titers from conjunctival swabs paralleled the course of infection and recovery was uneventful and complete within 7 days (9).

D. Experimental Infection of Avian and Mammalian Species

Since the influenza virus isolated from dead seals was antigenically similar to A/FPV/Dutch/27 (Hav1Neq1), it might be anticipated that the virus would replicate and perhaps cause disease in avian species. The virus was, therefore, inoculated into different avian and mammalian species for comparison (Table 1). The Seal/Mass/1/80 virus replicated poorly in avian species; low titers of virus were detected

Table 1. Recovery of A/Seal/Mass/1/80 (Hav1Neq1) from Experimentally Infected Mammals and Birds[1]

	No. animals infected[2] / No. animals inoculated	Days of virus[3] shedding
Mammalian		
Seals	4/4	10 (7.0)
Pigs	2/2	5 (6.5)
Cats	2/2	8 (6.5)
Ferrets	2/2	7 (8.0)
Guinea Pigs	2/2	6
Hamsters	0/3	0
Mice	6/6	5
Dogs	0/2	0
Avian		
Chickens	4/12	
Ducks	1/6	
Parakeets	1/2	
Turkeys	0/2	

[1]Mammals were inoculated intranasally with 10^6-10^8 EID_{50} of A/Seal/Mass/1/80; avian species, inoculated orally and intratracheally with 10^7 EID_{50}.
[2]Tracheal and cloacal samples were collected from avian species; nasal washes from dogs, cats, ferrets and guinea pigs. Lungs were taken from hamsters and mice. Samples were inoculated into 11-day-old embryonated eggs. Viruses were detected by hemagglutinin activity.
[3]Peak virus titer (log_{10} EID_{50}/ml of nasal wash) is given in parentheses.

in the respiratory tract of 1/3 of the baby chickens, 1/6 of the baby ducks, none of the turkeys and one of two parakeets. In no cases were viruses isolated from fecal samples and none of the avian species showed any disease signs.

The Seal/Mass/1/80 virus replicated to high titers in the respiratory tract of pigs, cats, and ferrets and was shed for 5 to 8 days post-infection (Table 1) with peak virus titers on day 3 p.i. However, these animals showed no signs of disease. The virus also replicated in guinea pigs and mice, but again, without signs of disease. No virus could be recovered from dogs or hamsters.

The above studies indicate that, despite the antigenic and genetic similarity of the A/Seal/Mass/1/80 virus to avian strains, the virus replicated better in mammalian, than in avian, hosts.

E. Genetic Studies

The above studies showed that 3 of the 7 structural proteins of the seal virus were antigenically similar to avian influenza viruses. The RNA segments of the seal influenza virus were, therefore, analyzed to determine the extent of genetic homology with influenza viruses from other species. The influenza virus strains chosen for comparison with the genes of the seal virus are listed at the top of Figure 1. These include representatives of all human, equine and swine subtypes and a series of avian influenza isolates from several species representing all avian hemagglutinin and neuraminidase subtypes.

RNA from each of these strains was used in competitive reassociation assays with individual labeled seal virus RNA segments coding for the nonsurface proteins (10). With all seal RNA segments, the most closely related corresponding RNAs were found in various avian influenza strains. For example, with RNA segment 5 (Figure 2), the most closely related strain was A/duck/Alberta/60/77. Although none of the strains tested contained genes closely related to all of the seal RNAs, the data clearly indicate that all of the genes of A/Seal/Mass/1/80 are closely related to genes from different avian viruses.

IV. DISCUSSION

We have reported the isolation and characterization of an influenza A virus possessing Hav1Neq1 [H7N7] surface antigens from seals that died of pneumonia on Cape Cod Pennisula in the winter of 1979-80. The high titers of virus in the lung tissues and the independent isolation of the virus in different laboratories (5,6) leaves no doubt that the virus originated from the seals.

The antigenic and genetic characterization of the A/Seal/Mass/1/80 virus shows that all of the RNA segments are most closely related to the RNAs from avian strains. Despite these relationships, the virus from seals is biologically similar to mammalian influenza viruses in its host

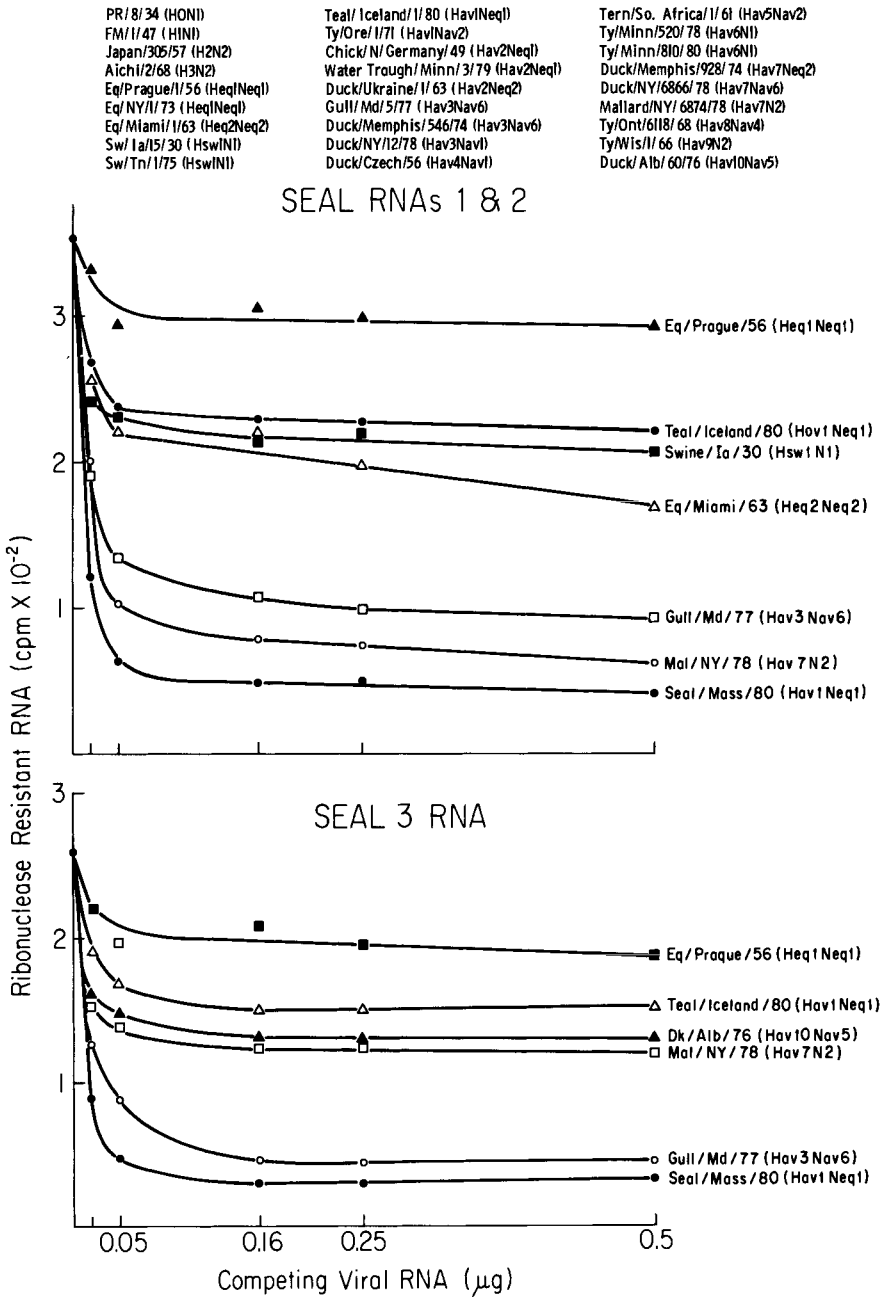
PR/8/34 (HONI)
FM/1/47 (H1N1)
Japan/305/57 (H2N2)
Aichi/2/68 (H3N2)
Eq/Prague/1/56 (HeqlNeql)
Eq/NY/1/73 (HeqlNeql)
Eq/Miami/1/63 (Heq2Neq2)
Sw/Ia/15/30 (HswlN1)
Sw/Tn/1/75 (HswlN1)
Teal/Iceland/1/80 (HavlNeql)
Ty/Ore/1/71 (HavlNav2)
Chick/N/Germany/49 (Hav2Neql)
Water Trough/Minn/3/79 (Hav2Neql)
Duck/Ukraine/1/63 (Hav2Neq2)
Gull/Md/5/77 (Hav3Nav6)
Duck/Memphis/546/74 (Hav3Nav6)
Duck/NY/12/78 (Hav3Navl)
Duck/Czech/56 (Hav4Navl)
Tern/So. Africa/1/61 (Hav5Nav2)
Ty/Minn/520/78 (Hav6N1)
Ty/Minn/810/80 (Hav6N1)
Duck/Memphis/928/74 (Hav7Neq2)
Duck/NY/6866/78 (Hav7Nav6)
Mallard/NY/6874/78 (Hav7N2)
Ty/Ont/6118/68 (Hav8Nav4)
Ty/Wis/1/66 (Hav9N2)
Duck/Alb/60/76 (Havl0Nav5)
SEAL RNAs 1 & 2
Eq/Prague/56 (Heq1Neq1)
Teal/Iceland/80 (Hov1Neq1)
Swine/Ia/30 (Hsw1N1)
Eq/Miami/63 (Heq2Neq2)
Gull/Md/77 (Hav3Nav6)
Mal/NY/78 (Hav7N2)
Seal/Mass/80 (Hav1Neq1)
SEAL 3 RNA
Eq/Prague/56 (Heq1Neq1)
Teal/Iceland/80 (Hav1Neq1)
Dk/Alb/76 (Hav10Nav5)
Mal/NY/78 (Hav7N2)
Gull/Md/77 (Hav3Nav6)
Seal/Mass/80 (Hav1Neq1)
Ribonuclease Resistant RNA (cpm X 10⁻²)
Competing Viral RNA (μg)
0.05
0.16
0.25
0.5

Figure 1. Competitive hybridization analysis of RNA gene segments of A/Seal/Mass/1/80 with influenza virus strains of avian and mammalian origin. Labeled RNA segments of A/Seal/Mass/1/80 were prepared as described (10) and annealed with homologous complementary RNA in the presence of increasing concentrations of RNA from the virus strains listed at the top of the figure.

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range, for it replicates to high titers in seals, ferrets, pigs and cats, replicates poorly in avian species, and does not replicate in the cells lining the intestinal tracts of ducks. It is not known whether the isolation of A/Seal/Mass/1/80 represents the recent introduction of this virus into seals from another species, or whether the pneumonia in seals resulted from perturbation of an established host-virus relationship that had previously not caused excess mortality. Retrospective and prospective studies are currently being done on seal sera stored in different countries in an attempt to obtain a more definitive answer to the above question. It is not clear how or when the seal influenza virus originated or why it is adapted to replicate in seals. Although we have not yet found an avian influenza virus containing all of the genes of the seal virus, such a strain presumably could have formed by reassortment since the exchange of genes among avian influenza viruses appears to be a frequent event (11). We do not know if this reassortment occurred in a bird or in a seal, following chance infection with two or more avian virus strains.

Although fowl plague virus (Hav1Neq1) has reportedly been isolated from man (12), serological evidence of infection was not obtained, leaving considerable doubt as to the origin of the virus. Recent studies suggest that avian influenza viruses can replicate in pigs and ferrets without producing disease (13,14). However, some avian viruses, such as A/tern/South Africa/61 (Hav5Nav2) and A/turkey/England/63 (Hav1Nav3) are virulent for laboratory mice and hamsters (15). These studies show that some avian influenza viruses can replicate and cause disease in mammalian species but there is no evidence for transmission within these species in nature.

Although the A/Seal/Mass/1/80 replicated and caused mild respiratory signs, eye infections and lung lesions in experimentally infected seals, none of the animals were as severely affected as in the field. The differences in severity may have been due to the absence of associated

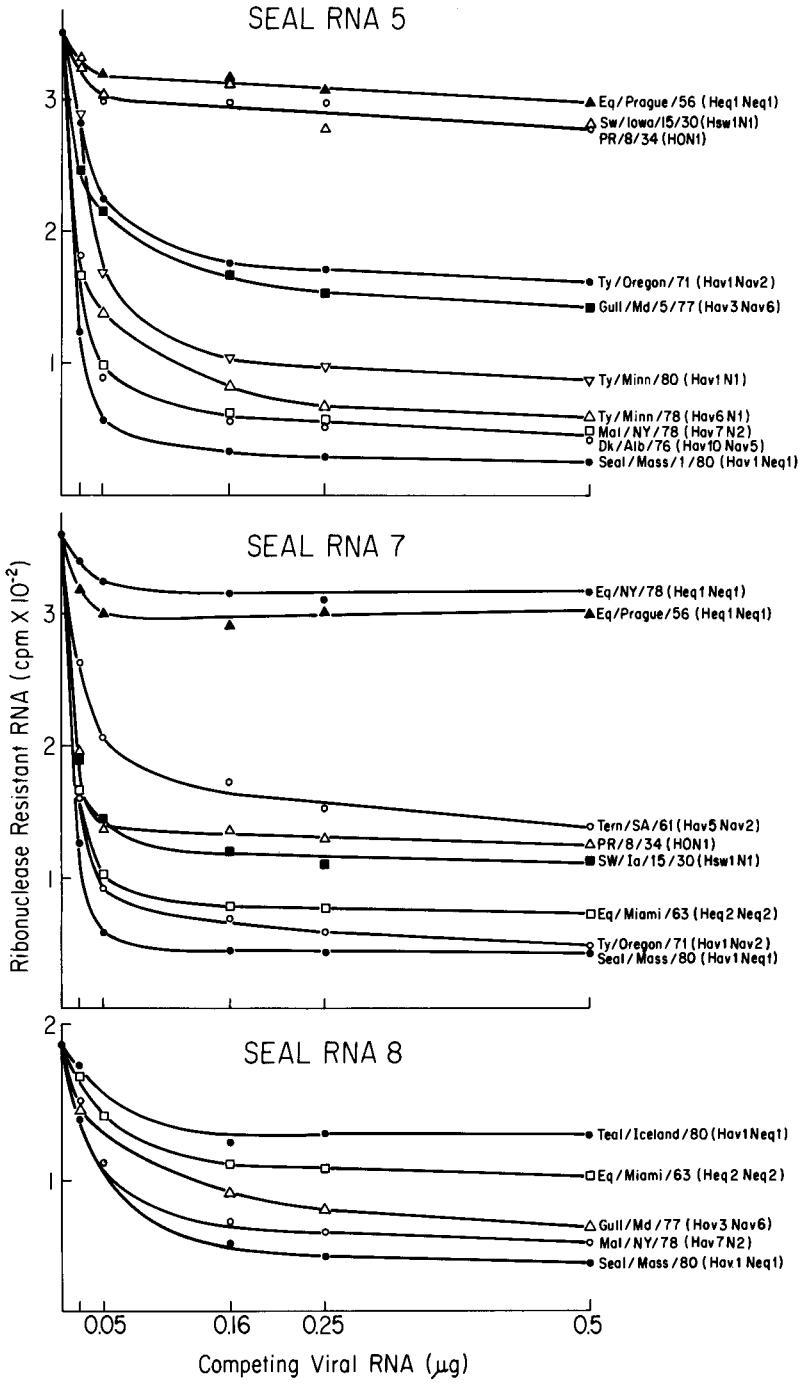
SEAL RNA 5
Eq/Prague/56 (Heq1 Neq1)
Sw/Iowa/15/30 (Hsw1N1)
PR/8/34 (HON1)
Ty/Oregon/71 (Hav1Nav2)
Gull/Md/5/77 (Hav3Nav6)
Ty/Minn/80 (Hav1N1)
Ty/Minn/78 (Hav6N1)
Mal/NY/78 (Hav7N2)
Dk/Alb/76 (Hav10Nav5)
Seal/Mass/1/80 (Hav1Neq1)
SEAL RNA 7
Eq/NY/78 (Heq1Neq1)
Eq/Prague/56 (Heq1Neq1)
Tern/SA/61 (Hav5Nav2)
PR/8/34 (HON1)
SW/Ia/15/30 (Hsw1N1)
Eq/Miami/63 (Heq2Neq2)
Ty/Oregon/71 (Hav1Nav2)
Seal/Mass/80 (Hav1Neq1)
SEAL RNA 8
Teal/Iceland/80 (Hav1Neq1)
Eq/Miami/63 (Heq2Neq2)
Gull/Md/77 (Hov3Nav6)
Mal/NY/78 (Hav7N2)
Seal/Mass/80 (Hav1Neq1)
Ribonuclease Resistant RNA (cpm X 10⁻²)
Competing Viral RNA (μg)
0.05
0.16
0.25
0.5

Figure 2. Competitive hybridization of RNAs 5, 7 and 8 of the seal virus. These RNAs code for the nucleoprotein, matrix and non-structural protein, respectively. Experimental details are given in the Legend to Figure 1.

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pathogens, such as the mycoplasmas that were isolated from the naturally infected seals (4), or because of the vast differences in environmental conditions that existed between the free-ranging and experimental seals at the time of infection. These factors may have also accounted for the presence of virus in the brains of only the naturally infected seals. The isolation of fowl plague influenza virus from the brains of mammals is of concern, for the seal influenza virus can replicate and cause conjunctival infection in humans (9), raising the possibility of further adaptation to humans.

V. ACKNOWLEDGEMENTS

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VI. REFERENCES

1. Easterday, B.C., In "The Influenza Viruses and Influenza" (E.D. Kilbourne, ed.), 449. Academic Press, New York, (1975).
2. Scholtissek, C., Curr. Top. Microbiol. Immunol. 80, 139, (1978).
3. Webster, R.G., and Laver, W.G., In "The Influenza Viruses and Influenza" (E.D. Kilbourne, ed.), 209, Academic Press, New York, (1975).

4. Geraci, J.R., St. Aubin, D.J., Webster, R.G., Madoff, S., Barker, I.K., and Prescott, J.R., (Submitted, 1981).
5. Lang, G., Gagnon, A., and Geraci, J., (Submitted, 1981).
6. Webster, R.G., Hinshaw, V.S., Bean, W.J., Van Wyke, K.L., Geraci, J.P., and Petursson, G., (Submitted, 1981).
7. Palmer, D.F., Coleman, M.T., Dowdle, W.R., and Schild, G.C., Immunology Series no. 6, (1981).
8. van Wyke, K.L., Hinshaw, V.S., Bean, W.J., and Webster, R.G., J. Virol. 35, 24, (1980).
9. Webster, R.G., Geraci, J.R., Pertursson, G., and Skirnisson, K., (Submitted, 1981).
10. Bean, W.J., Cox, N.J., and Kendal, A.P., Nature 284, 638, (1980).
11. Hinshaw, V.S., Bean, W.J., Webster, R.G., and Sriram, G., Virology 102, 412, (1980).
12. Campbell, C.H., Webster, R.G., and Breese, S.S., J. Infect. Dis. 122, 513, (1970).
13. Hinshaw, V.S., Webster, R.G., and Turner, B., J. Genet. Virol. 41, 115, (1978).
14. Ottis, K., and Bachmann, P.A., Arch. Virol. 63, 185 (1980).
15. Ulys, C.J., and Becker, W.B., J. Comp. Pathol. Ther. 77, 167, (1967).

INFLUENZA IMMUNOPROPHYLAXIS AFTER 30 YEARS' EXPERIENCE

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Public Health Service
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ABSTRACT

The summary report of the Secretary's Conference on Influenza, held July 26, 1978, states that "As a broad generalization, influenza vaccine can be expected to prevent infection and disease for 1-2 years after vaccination in about 7 of 10 people who receive it." The key word is "generalization." After 30 years, questions still persist about the efficacy of inactivated vaccines under certain conditions and in certain populations for whom the vaccine is recommended, particularly the elderly. Many questions related to efficacy stem from the inate antigenic and biologic variability of influenza viruses and the variable susceptibility of host populations. Other questions relate to the validity of the vaccine trial itself and the methods used to evaluate efficacy. In vaccine formulation emphasis has been placed on minimal dosages because of cost and undesirable toxic reactions. Newly emerging technology makes it possible to consider vaccines of significantly greater concentrations which may provide answers to many of the questions on vaccine efficacy.

At the Surgeon General's Meeting on Influenza convened on January 27, 1981, there were two presentations on influenza vaccine efficacy. The first was from Centers for Disease

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Control (CDC) staff members who, in cooperation with health officials from various states, had investigated the clinical efficacy of influenza vaccine in four nursing homes and other institutions where influenza-like outbreaks had occurred during the 1980-81 season. In each outbreak A/Bangkok/80 (H3N2) was responsible for at least some of the illnesses. This was demonstrated by virus isolation or serologic evidence of infection during or shortly after the outbreak. The average vaccine efficacy, based on retrospective studies of these outbreak, was approximately 30 percent--a disappointingly low figure when one recognizes that the H3N2 antigens in the vaccine were essentially identical to those of the virus which caused the outbreak.

The second presentation was given by Dr. William Barker, who reviewed data from a recent report on the effect of influenza vaccination on reduction in pneumonia and influenza hospitalizations and deaths among elderly members of the prepaid health plan (1). Drs. Barker and Mullooly reported that, during the 1972-73 epidemic, vaccine derived from the A/Hong Kong/68(H3N2) virus was associated with an estimated 72 percent reduction in hospitalization and an 87 percent reduction in mortality. This high rate of efficacy was observed in spite of the rather significant antigenic drift of the epidemic strain A/England/42/72, away from the vaccine strain. These two differing reports came as no surprise. In fact, such diametrically opposite conclusions at influenza vaccine meetings are rather commonplace.

In reference to the CDC presentation, Dr. Walter Orenstein, the epidemiologist reviewing the data, prefaced his remarks by reminding the audience that: 1) the population at risk in all four studies was small, 2) all residents could not be said to have had equal exposure to influenza virus, 3) most influenza-like illnesses were not confirmed by laboratory diagnosis, and 4) the 95 percent confidence limits of vaccine efficacy ranged from 0-57 percent to 0-76 percent. Concerning the second presentation, it should be noted that many of the people 65 years of age or older produced exceptionally high serum antibody responses to the A/Hong Kong vaccine, because they had been exposed to an antigenically similar strain during epidemics shortly after the turn of the century (2). In the first presentation the bias is toward vaccine failure; in the second, it is toward vaccine efficacy.

The first evidence of influenza vaccine efficacy was obtained during the winter of 1935-36 by Stokes and coworkers (3). Remarkably, these data were available only 2 years after the influenza virus was first isolated from specimens from humans. Even more remarkably, the vaccine shown to be efficacious consisted of an active virus filtrate from a

10 percent emulsion of infected mouse lung in normal saline. Approximately one-sixth of the inmates in an institution for men and boys were injected subcutaneously with swine virus and another sixth were injected with the virus isolate from humans. About 25 percent of the inmates developed respiratory infections within weeks after vaccination. The incidence of febrile infections was approximately 12.5 percent for controls and for persons vaccinated with swine influenza virus. The incidence of febrile disease was 2.7 percent among human virus vaccine recipients, giving an efficacy rate of approximately 78 percent. The authors concluded that intramuscular vaccination with active human influenza virus was protective. They also noted that resistence to infection and the neutralizing properties of serum were associated, but not always parallel. No final conclusions on the value of influenza immunization were drawn, but further studies among larger groups of people were proposed.

Within the next several years, well over a half-dozen vaccine studies were begun, but findings in respect to antibody response or protection were inconsistent (4). It was during this period that experiments by Francis (5) demonstrated the importance of concentrated virus in effective vaccines. The dose of vaccine was shown, in mice, to be directly proportional to the degree of protection against infectious challenge of vaccinated animals.

The first major field trials with concentrated influenza vaccine began in 1942. The Commission on Influenza, under the Armed Forces Epidemiological Board, vaccinated groups in various state institutions with vaccines concentrated by a variety of means. True to form, there was no influenza epidemic in 1942. The next year, the study was repeated with a controlled experiment in Army student training units being set up in nine universities throughout the country. By early December, 1943, over 6,000 students had been vaccinated subcutaneously with 1 ml of 10 x polyvalent vaccine consisting of formalinized concentrates of alantoic fluid eluates of A/PR8, A/Weise, and B/Lee. Over 6,000 controls received a placebo. Shortly after the vaccination program was completed, epidemics of influenza A occurred in all of these institutions. The incidence of influenza-like disease was approximately 70 percent less in the vaccinated than in the unvaccinated (6).

In brief, by 1945, the general consensus was that the vaccine was efficacious for both influenza A and influenza B viruses, but that efficacy depended upon vaccine potency, antigenic relevance, and administration within 1 to 6 weeks before onset of the epidemic.

Thereafter, the Commission on Influenza of the Armed Forces Epidemiological Board and the Medical Research Council

of Great Britain sponsored numerous field trials with inactivated vaccines (7). In 1962, Fred Davenport (8) summarized results of 22 field trials that the Commission on Influenza had carried out in military populations during the previous 2 decades. He reported that in 18 successful influenza A field trials the average protection was 78 percent, with a range from 41 to 94 percent. In four field trials against influenza B, the average protection was 90 percent, with a range from 63 to 96 percent. These figures, of course, did not include the years of 1947, 1955, and 1957, when major antigenic drifts or shifts rendered the vaccine ineffective.

Not all of the data from the Commission's trials are readily available for review, but published reports consistently demonstrate a reduction of febrile respiratory disease among military age vaccinees during epidemic periods (9)(10). The Commission on Influenza no longer exists. It was disbanded partly because, in the words of one longtime member, "influenza vaccine efficacy was demonstrated with monotonous regularity in the military for nearly 30 years."

Not so for civilians (11). The differing findings of civilian and military vaccine studies generated considerable controversy during the mid-1960's and raised questions about recommendations for vaccine use in civilian populations (12) (13). Considering the large number of variables that may influence any influenza vaccine trial, controversy should not be surprising.

Variations in vaccine efficacy among civilian populations have even been reported from trials conducted during the same period by the same investigators using similar vaccines produced by the same manufacturer. Variables which may influence vaccine efficacy include vaccine potency, the vaccine's antigenic relevance, the vaccinees' previous antigenic experience and age, the opportunities for virus transmission, length of surveillance, method of surveillance, background respiratory disease, laboratory confirmation of infection, and the sensitivity of laboratory tests. There are, no doubt, other variables, of which we are unaware. It is unlikely that we will ever be able to assign a percent efficacy to influenza vaccine which would apply under all conditions and in all situations.

In July, 1979, at a workshop on influenza in Bethesda, Maryland, Dr. Robert Couch reviewed the results of inactivated influenza vaccine field trials published in scientific journals printed in the English language. He reported 71 field trials, but reminded participants that even this list was incomplete. The range of reported effectiveness for influenza virus types A and B was 0 to 96 percent. Against homologous virus challenge, effectiveness was reported to be

greater than 60 percent. Protection against heterologous virus was more variable. Greater protection was reported for studies involving military personnel than civilians; this was particularly notable for type B vaccines. He concluded that his review of the scientific literature provided support for the belief that influenza vaccines are generally effective for prevention of clinical influenza, particularly if the epidemic virus is antigenically similar to the vaccine virus and if vaccine is given in the few months immediately preceding exposure. Under these conditions, he stated, the commonly quoted figure of 70 percent vaccine efficacy is reasonable.

This, generally, has been the view of the Immunization Practices Advisory Committee (IPAC), which for nearly 20 years has recommended annual vaccination for all individuals considered to be at increased risk of adverse consequences from infections of the lower respiratory tract. Included in this recommendation are older persons, particularly those over age 65. Over the years the IPAC has been sometimes more and sometimes less permissive in recommending vaccination of healthy persons under age 65, persons who provide essential community services, and persons who may be at increased risk of exposure, such as medical care personnel. The reluctance to recommend vaccination for everyone stems from the assumed need for annual vaccination and the observation that excess mortality in influenza occurs primarily among those who have underlying illness or are over age 65 (14).

The IPAC recommendations are based on two important assumptions; namely, that 1) annual vaccination is effective, and 2) vaccine efficacy for the elderly and infirm is similar to that reported for the young and healthy military recruit. Several observations have cast doubt on these two assumptions.

In 1979, Hoskins and coworkers (15) reported the effect of annual vaccination with inactivated influenza vaccine in a boys' boarding school during outbreaks in 1972, 1974, and 1976. In each outbreak the vaccine provided protection to those boys who had not had previous experience with H3 viruses and who were vaccinated for the first time with the most up-to-date strain. In later epidemics revaccination with the same strain did not increase the degree of protection and revaccination with a later strain did not provide protection against subsequent challenge. In short, by the end of the third epidemic the number of subjects having influenza over the 5-year period was similar, regardless of their vaccination history. Sparks (16), in 1979, reported similar findings in a boys' school after successive epidemics of influenza A. These reports are largely unsubstantiated, but they raise questions which must be answered.

Despite the large number of trials which have been performed since 1935 in this country, the question about the long-term efficacy of annual vaccination has not been specifically addressed. The military studies have primarily involved recruits for periods of less than 1 to 2 years. Although annual vaccination is privided for all military personnel, the high personnel turnover and movement from base to base preclude long-term studies. In Japan, annual vaccination is practiced through the immunization of 18 million persons of school age with at least one dose of vaccine (17), but even in Japan, firm data on the effect of annual vaccination are not available.

As I stated earlier, the second assumption which remains unproven is that the efficacy of influenza vaccine in the elderly and infirm is comparable to that seen in young and healthy military recruits. D'Allessio and coworkers (18) found that influenza vaccines given in 1966 and 1967 provided no protection against an outbreak of H2N2 virus in a county home for the aged during the winter of 1967-68. The outbreak occurred just 4 weeks after the second of two doses of influenza vaccine had been given. Despite adequate documentation of influenza infection by virus isolation and serologic titer rises, no relationship was seen between vaccination and incidence of disease.

However, Stuart et al. (19) reported evidence of vaccine efficacy in a study performed in 1965 and 1966 on the effects of monovalent adjuvant and subsequent aqueous influenza vaccines. Highest vaccine efficacy (approximately 96 percent) against febrile respiratory disease occurred when persons who were given adjuvant vaccine in 1964 received aqueous vaccine just before the outbreak in 1965-66. Monovalent adjuvant vaccine given more than a year before the outbreak appeared to protect as well as (55 percent), if not slightly better than, monovalent aqueous vaccine (38 percent) administered several months before the epidemic to people without prior benefit of adjuvant vaccine. These results are often quoted as demonstrating 96 percent protection in the elderly with influenza vaccines, but this figure may not be appropriate for aqueous vaccines because of the population's experience the year before with adjuvant vaccines.

A review of eight of the most thoroughly planned prospective vaccine trials in geriatric populations shows a reported protection rate ranging from 0 to 77 percent, with a mean of 38 percent (18-25). This figure does not include the previously described retrospective study of Barker and Mullooly (1), in which they found a 72 percent efficacy rate. If only those trials in which the HA antigen in the vaccine is closely related to or identical to that of the epidemic strain are

considered, the mean is 49 percent. Interestingly, in several trials where vaccine in the elderly populations was reported to be disappointing, efficacy for the same or similar vaccines in younger populations was reported to be protective (20, 21, 25).

Several explanations for the apparent lower vaccine efficacy among geriatric populations have been proposed. One is that it reflects their decreased immunologic competency. Another is that the epidemiology of influenza is peculiar to institutions. Still another is that annual immunization may provide no long-term protective advantage to the elderly. There may be a simpler explanation, that is, that studies in geriatric populations have been few and that for ethical considerations, few of these studies have included true control groups. Whatever the reason and whatever the interpretation, the fact remains that we do not have consistent evidence of influenza vaccine efficacy in elderly populations.

We know that natural immunity is protective--at least for a longer period than that attributed to inactivated vaccines. The influenza epidemics that occur for the 5 to 6 years after a new variant is introduced, as in 1957 and 1968, do not affect the same individuals (26). Natural protection may extend for 3 to 10 years or more. Perhaps the most extreme example of long-term natural protection is that afforded against the current H1N1 strains by previous exposure to these viruses nearly 30 years ago.

Dr. Francis' demonstration in 1939 that protection against influenza in mice could be correlated with vaccine dose helped considerably to bring some order out of the chaos generated by the successes and failures of the early trials. The preparation of vaccines with concentrated virus made it possible to go on to the successful field trials of 1943 and to raise the level of confidence in vaccination against influenza. Perhaps we did not go far enough.

The virus concentrations used in 1943 and those used now do not produce maximum circulating or secretory antibody titers in vaccine recipients. Our present practice of judging a vaccine by its ability to stimulate circulating antibody titers of $\geq$1:40 is recognized to be a minimum requirement.

Until now, the constraints to the use of vaccine with higher antigenic content have been cost and adverse reactions. As Dr. Salk stated in the mid-1940's, "From these points of view, therefore, the desirability of considering how little virus may be used is clearly indicated." In the 1980's we are entering an era during which we may be able to produce antigens through recombinant DNA or synthetic means, cheaply, in large quantities, and without extraneous viral proteins. Perhaps we are approaching the time when the desirability

of considering how much antigen may be used is clearly indicated.

In recent years, concentrated inactivated polio vaccines have been shown to be just as effective or, in some instances, more effective, than live vaccine. Inactivated measles vaccine, when prepared properly, may be as effective as live vaccine. Concentrated and inactivated rabies vaccines have provided a measure of protection heretofore unachieved. We need to reexamine our attitudes toward the concept of inactivated influenza vaccines and reexplore the effects of substantially greater antigen concentrations.

Many studies on the effects of vaccine dosage on serum antibody response have been described, but three are particularly worth mentioning. Salk (27) demonstrated in 1948 that increasing concentrations of vaccine produced increasing serum antibody titers. Antibody titers continued to increase even with doses up to approximately 2,000 CCA units, the highest administered. The smaller the dose, the smaller the increase in antibody titers and the more rapidly antibody titers returned to initial levels. Antibody titers resulting from the highest (2,000 CCA) dose decreased with time, but remained high even after 1 year. Mostow and coworkers (28) reported a similar finding for zonal purified influenza vaccine with graduated doses up to 4,800 CCA units, the highest concentration used. There was no point at which a further increase in antigen concentration failed to produce a further improvement in serum antibody response, even against distantly related heterologous antigens. Neutralizing activity in nasal washings also correlated directly with vaccine dosage and serum antibody titers. Eighty percent of subjects receiving the 4,800-CCA unit vaccine had evidence of neutralizing activity in nasal secretions, but none in the placebo group had such activity. Like Salk, they found a 2- to 3-fold increase in geometric mean titers of serum antibody for each 10-fold increase in vaccine concentration. Ruben and Jackson (29) reported a similar experience with disrupted tri-(n-butyl)-phosphate vaccine. The highest dose, 6,400 CCA units of vaccine, produced a nearly 75-fold increase in serum antibody geometric mean titer.

Field trials with highly concentrated vaccines have been few. We may reasonable assume, however, that substantial increases, that is 10-fold or more, over the present vaccine dosage should result in substantially greater and longer protection against influenza (30). In at least one field trial (24) a 10-fold increase in antigen concentration (up to 3,000 CCA units) essentially doubled the clinical efficacy of the vaccine against influenza A(H3N2).

The possibility that new influenza vaccines may be derived from proteins produced through recombinant DNA or synthetic techniques suggests an opportunity not only to introduce newer methodologies for vaccine production, but also to provide a safe and highly effective vaccine for general use. Using highly concentrated vaccines may overcome concern about minor antigenic drift in epidemic strains and the need for annual immunization, the two criticisms which cause us to limit our vaccine recommendation to those who are at the highest risk of death. We are at the very beginning of a third generation of influenza vaccines. We know nothing at this point regarding the immunogenicity, toxicity, or feasibility of polypeptide antigens, but they are highly appealing. They provide the first opportunity of going beyond the vaccine barrier we have faced since 1939.

REFERENCES

1. Barker, W.H., and Mullooly, J.P., JAMA 244, 2547 (1980).
2. Masurel, N., and Marine, W.M., Am. J. Epid. 97, 44 (1973).
3. Stokes, J., Jr., Chenoweth, A., Waltz, A., Gladen, G., and Shaw, D., J. Clin. Invest. 16, 237 (1937).
4. Blake, F.G., Bull. N.Y. Acad. Med. 24, 308 (1948).
5. Francis, T., Jr., J. Experimental Med. 69, 883 (1935).
6. Commission on Influenza, Armed Forces Epidemiological Board, Office of Surgeon General, U.S. Army, JAMA 134, 982 (1944).
7. Medical Research Council Committee on Influenza and Other Respiratory Virus Vaccines, British Med. J. 1, 415 (1958).
8. Davenport, F.M., JAMA 182, 11 (1962).
9. Mogabgab, W.J., and Leiderman, E., JAMA 211, 1672 (1970).
10. Stiver, H.G., Graves, P., Eickhoff, T.C., and Meiklejohn, G., NEJM 289, 1267 (1973).
11 Bashe, W.J., Stegmiller, H., Leonida, D., and Greenwald, P., NEJM 270, 870 (1964).
12. Langmuir, A.D., Henderson, D.A., and Serfling, R.E., Am. J. Public Health. 54, 563 (1964).
13. Davenport, F.M., Med. J. Australia 1, Supplement 1, 33 (1973).
14. Center for Disease Control, MMWR 29, 225 (1980).
15. Hoskins, T.W., Davies, J.R., Smith, A.J., Miller, C.L., and Allchin, A., Lancet 1, 33 (1979).
16. Sparks, J.P., Lancet 1, 317 (1979).
17. Dowdle, W.R., Millar, J.D., Schonberger, L.B., Ennis, F.A., and LaMontagne, J.R., J. Infect. Dis. 141, 258 (1980).
18. D'Allessio, D.J., Cox, P.M., and Dick, E.C., JAMA 210, 485 (1969).

19. Stuart, W.H., Dull, H.B., Newton, L.H., McQueen, J.L., and Schiff, E.R., JAMA 209, 232 (1969).
20. Edmondson, W.P., Jr., Rothenberg, R., White, P.W., and Gwaltney, J.M., Jr., Am. J. of Epid. 93, 480 (1971).
21. Ruben, F.L., Johnston, F., and Streiss, E.J., JAMA 203, 863 (1974).
22. Serie, C.,Barme, M., Hannoun, C., Thibon, M., Bech, H., and Aquino, J.P., Develop. of Biological Standards 39, 317 (1977).
23. Aymard, M., Bentejac, M.C., Larbaigt, G., Michaut, D., and Triau, R., Develop. of Biological Standards 43, 231 (1979).
24. Schoenbaum, S.C., Mostow, S.W., Dowdle, W.R., Coleman, M.T., and Kaye, H.S., Bull. WHO 41, 531 (1969).
25. Feery, B.J., Evered, M.G., and Morrison, E.I., J. Infect. Dis. 139, 237 (1979).
26. Fry, J., British Med. J. 1, 745 (1961).
27. Salk, J.E., J. Immunol. 58, 369 (1948).
28. Mostow, S.R., Schoenbaum, S.C., Dowdle, W.R., Coleman, M.T., Kaye, H.S., and Hierholzer, J.C., Am. J. Epid. 92, 248 (1970).
29. Ruben, F.L., and Jackson, G.C., J. Infect. Dis. 125, 656 (1972).
30. Dowdle, W.R., Mostow, S.R., Coleman, M.T., Kaye, H.S., and Schoenbaum, S.C., Postgraduate Med. J. 49, 159 (1973).

THE BASIS FOR IMMUNITY TO INFLUENZA IN MAN[1]

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I. ABSTRACT

Infection or vaccination with an influenza virus induces a variety of humoral and cellular immune responses. Among these, the subtype specificity and duration of antibody responses most closely match related characteristics of infection-induced immunity in man. Furthermore, *in vitro* and *in vivo* animal studies suggest that the most probable mediator of resistance to influenza is antibody to the hemagglutinin (HA), either in serum or respiratory secretions. A role for anti-HA antibody in secretions was sought in human studies utilizing immunization followed by virus challenge and sensitive radio-immunoprecipitation assays for detection of antibody. Serum IgG antibody was more consistently and more strongly related to resistance to challenge than was antibody in secretions. Moreover, passive immunization of infants has been recently shown to be associated with immunity to influenza. Thus, we believe currently available evidence favors the concept that serum IgG antibody to the influenza virus hemagglutinin is not only the best correlate but is probably the primary mediator of immunity to influenza.

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II. INTRODUCTION

Infection or vaccination with an influenza virus induces a variety of humoral and cellular immune responses. The first described and most commonly used immune correlate to resistance to infection is serum antibody to the virus as measured by hemagglutination inhibition or neutralization tests (1). Recent descriptions of more sensitive methods for measuring immunoglobulin-specific classes of antibody to the virus and its components and of several cell mediated immune functions in infected animals provide a reason for reevaluating the basis for immunity to influenza in man. This report constitutes such a reevaluation.

III. RESULTS

A. Candidate Mediators and/or Mechanisms for Immunity

That immunity to infection with influenza virus can be complete has been amply demonstrated in studies involving challenge of human volunteers (2). Mediators and/or mechanisms accounting for this immunity should exhibit a duration and specificity similar to that observed in man. The duration of immunity to reinfection with homologous virus was shown in challenge studies to be greater than four years (3). Because of the absence of cross reacting antibodies to the hemagglutinin (HA) surface antigens of different subtypes of type A viruses and the documented occurrence of widespread infection and disease with change in prevalence of subtype, immunity to type A is considered to be subtype specific (4).

We recently examined the heterotypic immunity to type A viruses by rechallenging volunteers who had documented prior infection with a specific type A/H3N2 virus. It was observed that heterotypic immunity apparently spanned the degree of antigenic variation represented by the H3N2 variants detected between 1968 and 1975, i.e., A/Hong Kong/68, A/England/72, A/Port Chalmers/73, and A/Scotland/74 viruses (unpublished data). However, the immunity apparently did not extend to A/Victoria/75. Thus, immunity may not completely span a subtype of type A virus. A similar inability for immunity to span all degrees of antigenic variation within a subtype must surely occur for type B viruses.

The varied immune responses to influenza virus infection of experimental animals are listed in Table I. Differences in duration and specificity of some immune factors have been described for animals who were primed by inactivated virus,

but current considerations for man relate only to priming by infection. In view of the subtype specificity of the immunity induced in man by influenzal infection, only those mediators or mechanisms that exhibit subtype/variant specificity are primary candidates for mediating immunity to infection. Although some authors have described subtype specificity for T-cell mediated cytotoxicity in infected mice, most authors have primarily detected type specificity (11-13, 15). Similarly, studies in man have described only type specificity (14). Moreover, since T-cell cytotoxicity does not develop in infected animals until after infection has occurred, it is not a major candidate for mediating resistance to infection. Thus, although a variety of immune mechanisms have been identified that might participate in recovery from infection, among those shown, only antibody mediates subtype/variant specific immunity that persists for periods of time similar to those described in man.

TABLE I. Time of Appearance and Specificity of Immune Mediator or Mechanism Described in Animals Previously Infected with Type A Influenza Virus

Present before reinfection (remotely after prior infection)
No specificity
Macrophage mediated viral inactivation (5)
Type specificity
Lymphocyte blastogenesis (6)
Skin sensitivity (7)
Antibody to matrix and RNP proteins (8)
Subtype/variant specificity
Antibody dependent cellular cytotoxicity (9)
Antibody to hemagglutinin and neuraminidase proteins (4)
Present early after infection
No specificity
"Activated" macrophages (5)
Antiviral effects of interferon
Natural killer cell cytotoxicity (10)
Type specificity
T-cell cytotoxicity (11, 12, 13, 14)
Subtype/variant specificity
T-cell cytotoxicity (15)
Antibody dependent cellular cytotoxicity (16)
Antibody to hemagglutinin and neuraminidase proteins (4)

Described mechanisms for antibody mediated resistance to virus infection are interference with adsorption, aggregation and clearance of virions, opsonization for phagocytosis, antibody-complement mediated lysis of infected cells, and antibody dependent cell mediated cytotoxicity. Although the latter might be considered a cellular immune mechanism, cytotoxicity is not demonstrable when all free and cell bound antibody are removed from the assay system (9). For this reason, it can also be considered an antibody mechanism.

Antibody to the neuraminidase subunit (NA) has been shown to reduce the quantity of infectious virus detected in infected cultures and animals (17). It is not capable of preventing infection unless present in high titer (18).

Thus, the major antibody responsible for preventing infection is that directed against the HA, and the presence of a sufficient concentration of antibody to HA in serum has been the most consistent correlate of immunity to influenza in both man and animals. The question has been "Is this antibody the mediator or merely a correlate of the primary mediator of resistance to infection with influenza virus?" Since no other mediators or mechanisms have been described that are significant candidates for a primary role in resistance to influenza, the question becomes whether serum or secretory anti-HA antibody is the primary mediator of resistance to infection.

B. Serum Versus Secretion Antibody

About 40 years ago, Francis proposed that the primary role for circulating antibody to influenza virus was to control local spread of infection and to prevent severe disease, whereas prevention of infection was accomplished by antibody in respiratory secretions (19). Proof for this thesis was not provided for man, but supporting data were provided by Fazekas de St. Groth utilizing experimental infections in mice (20). Since that time, a prevalent concept has been that antibody in respiratory secretions is the primary mediator of resistance to infection. Since the major immunoglobulin in secretions is IgA containing secretory piece (sIgA), it has been assumed that this antibody is the major mediator of resistance to influenza. Our efforts to support this thesis have centered on studies in normal volunteers experimentally challenged with virus. Although not generally performed primarily for that purpose, results after challenge of immunized volunteers have been evaluated for a relation between antibody location and occurrence of infection. When tested by neutralization tests, a variable number of volunteers who develop serum antibody to influenza will also develop detectable antibody in concen-

trated nasal wash specimens. Examples of the type of result generally obtained are shown in Table II. Represented are four studies, two with challenge after vaccination with inactivated vaccine intramuscularly (IM), and two after prior infection. When tested in neutralization tests, persons with antibody detected only in secretions are uncommon and thus do not represent a category for comparison.

TABLE II. Relation of Location of Antibody to Occurrence of Infection with Influenza Virus

Group		Prior inactivated vaccine		Prior infection	
Serum Ab[a]	NW Ab[a]	Study 1	Study 2	Recent	Remote
Absent	Absent	20/29[b]	11/15	9/9	29/40
Present	Absent	4/18	2/15	3/12	1/25
Present	Present	8/29	0/17	0/4	2/26

[a]As determined in tissue culture neutralization tests against 10-60 $TCID_{50}$ of virus.
[b]No. infected/no. in group.

In each study shown in Table II, among volunteers with no detectable antibody in secretions, significantly fewer infections occurred among persons with antibody in serum than among those with no detectable antibody. Although two of the studies suggested an additional benefit if antibody was also detected in secretions, in no instance was the frequency of infection significantly lower among those with antibody in both locations than among those with serum antibody only. The failure to find evidence for a primary role of antibody in secretions is generally assumed to be because the rapid turnover of antibody in this location makes detection difficult with relatively insensitive serologic methods. Thus, despite the consistent finding of a relationship between serum antibody and resistance to influenza and the lack of evidence of a primary role for antibody in secretions, the belief that sIgA is a primary mediator of resistance to influenza has remained prominent.

We recently developed sensitive radioimmunoprecipitation (RIP) procedures for quantitating IgG and sIgA directed against the influenza virus HA (21, 22). These assay proce-

dures were used for reassessing the role of serum and secretory antibody in resistance to influenza. Studies thus far analyzed involve (1) challenge with A/Scotland (H3N2) virus after IM vaccination with placebo, inactivated whole virus, or purified HA and NA subunits of A/Scotland virus; (2) challenge with A/Victoria (H3N2) virus after intranasal vaccination with placebo, live attenuated cold adapted, or temperature sensitive A/Victoria vaccine; and (3) challenge with A/USSR (H1N1) virus after placebo, IM inactivated whole virus vaccine, or live attenuated cold adapted A/USSR vaccine intranasally. Just prior to challenge, all volunteers had antibody to the challenge virus quantitated in serum for RIP IgG and for neutralizing activity, and in secretions for RIP IgG, RIP sIgA, and neutralizing activity. The starting dilution for serum RIP tests was 1:50, and endpoints were expressed as that dilution of serum which precipitated 20% of the radiolabeled HA upon addition of antiserum to IgG. For secretions, the initial dilution for RIP tests was 1:20 of 10- to 20-fold concentrated nasal wash specimens. Concentration of the nasal wash specimens was required for detection of neutralizing antibody, but dilution was necessary for technical reasons in the performance of RIP tests. For secretions, greater than 5% precipitation of radiolabeled HA by the initial dilution upon addition of antiserum to secretory component (i.e., sIgA) or IgG represents significant antibody. Occurrence of infection was assessed by testing sequential specimens of nasal secretions for virus shedding and serum for rise in specific antibody as previously described (23).

The relationship between titer of serum RIP IgG after IM vaccination with A/Scotland vaccine (H3N2) and occurrence of virus shedding and infection is shown in Table III. The fre-

TABLE III. Relationship of Serum IgG Antibody after In-Activated A/Scot (H3N2) Vaccine to Infection and Illness after Challenge with Live A/Scot Virus.

Serum RIP IgG Ab[a]	No. vol.	No. who shed virus	No. Infected
<50-200	11	8	8
200-400	2	1	1
400-1000	9	2	4
≥1000	21	0	0

[a]Reciprocal of titer as determined by radioimmunoprecipitation.

quency of volunteers with virus shedding and infection (virus shedding and/or antibody titer rise) decreases significantly with increasing antibody titer (Wilcoxon, $p<.01$). A similar analysis for serum neutralizing antibody titers and nasal wash neutralizing titers (data not shown) was also significant ($p<.01$ for each antibody site). Finally, when tested for RIP sIgA or RIP IgG in secretions, presence of each of these antibodies was associated with significantly fewer infections than occurred among those without these antibodies. Thus, detection of antibody in any location by any of the serologic procedures was associated with reduced infection. Although no discriminating ability was provided by results of this study, the results do demonstrate a broad humoral immune response to inactivated vaccine in persons primed by infection.

The relationship between serum RIP IgG titers and virus shedding and illness for the volunteers immunized with A/Victoria (H3N2) and A/USSR (H1N1) vaccines are shown in Tables IV and V. Significantly fewer persons exhibited virus shedding and infection with increasing serum RIP IgG antibody for those given A/Victoria vaccines (Wilcoxon, $p<.05$), but for A/USSR vaccines significance was limited to virus shedding (Wilcoxon, $p<.05$). For each study, serum neutralizing antibody (data not shown) was associated with reductions in both infection and virus shedding. Thus, for all three studies, increasing serum neutralizing and serum RIP IgG antibody titers were significanly associated with reducing evidence of infection.

TABLE IV. Relationship of Serum IgG Antibody after Attenuated A/Victoria (H3N2) Vaccine to Infection and Illness after Challenge with Wild A/Victoria Virus.

Serum RIP IgG Ab[a]	No. vol.	No. who shed virus	No. infected
<50-200	4	4	4
200-400	6	3	5
500-1000	7	3	3
≥1000	8	0	0

[a]Reciprocal of titer as determined by radioimmunoprecipitation.

TABLE V. Relationship of Serum IgG Antibody after Attenuated or Inactivated A/USSR (H1N1) Vaccine to Infection and Illness after Challenge with Live A/USSR Virus.

Serum RIP IgG Ab[a]	No. vol.	No. who shed virus	No. infected
<50-200	9	8	8
200-400	8	4	5
400-1000	5	0	0
≥1000	4	0	2

[a]Reciprocal of titer as determined by radioimmunoprecipitation.

When assessed for antibody in secretions, a consistent relationship between an antibody assay and resistance to infection was detected only for RIP IgG antibody in secretions (Fisher exact, p=.04 and .03 for A/Victoria and A/USSR studies, respectively). Neither neutralizing nor RIP sIgA antibody in secretions was associated with resistance to infection in the A/Victoria studies, and the presence of RIP sIgA was also not significantly associated with resistance to A/USSR challenge (neutralizing titers for secretions were not available for this study). Thus, neither neutralizing antibody nor the presumed major antibody in secretions, secretory IgA, were associated with resistance to infection in these two studies. Somewhat surprising, however, was the finding of a clear relationship of IgG in secretions to resistance.

The occurrence of infection according to location of antibody is summarized in Table VI for the three studies presented above. Neutralizing antibody is used in the table to categorize presence or absence of antibody in serum, since most persons had cross reacting antibody to H3N2 and H1N1 at the time of studies and possessed some serum RIP IgG antibody. The presence of either RIP sIgA or RIP IgG is used to designate presence of antibody in secretions. (When neutralizing antibody in secretions was used, results similar to those in Table II were detected.) In each study, significantly fewer infections were detected among those with antibody in both locations than for those with neither serum nor secretion antibody (Fisher exact tests, p<.01 for A/Scotland and A/Victoria, .01 for A/USSR). Significantly fewer infections

also occurred for those in the A/Scotland study with antibodies in both locations than for those possessing secretion antibody only (Fisher exact, p=.05), and significantly fewer persons exhibited viral shedding in the A/USSR study if only serum antibody was present than if neither antibody was present (data not shown; Fisher exact, p=.05). Comparisons between other groups did not reveal significant differences. When only one immunoglobulin class of antibody in secretions was used to categorize volunteers, the presence of antibody in both locations remained associated with significantly fewer infections than occurred in those with neither antibody; however, significantly fewer volunteers developed infection among those with serum antibody only than among those with neither antibody in the A/Victoria study (Fisher exact, p<.01).

TABLE VI. Relation of Location of Antibody to Occurrence of Infection with Influenza Virus

Group		Study		
Serum Ab[a]	NW Ab[b]	A/Scot	A/Vic	A/USSR
Absent	Absent	7/10[c]	8/9	6/6
Absent	Present	4/4	1/1	4/6
Present	Absent	1/4	3/8	3/5
Present	Present	1/25	0/7	2/9

[a]As determined in tissue culture neutralization tests against 10-60 $TCID_{50}$ of the indicated virus.
[b]As determined in radioimmunoprecipitation tests.
[c]No. infected/no. in group.

The apparent primary significance of serum IgG antibody in the above described studies is supported by recent findings on the significance of passively acquired antibody in man. In a prospective survey, hospitalized infants infected with type A influenza were matched with cord blood antibody titers present at birth. A significant positive correlation was detected between the titer of cord blood antibody and the age of infection of the infant, suggesting a protective effect of the passively acquired antibody (24).

IV. DISCUSSION

In the present report, we assessed the status of knowledge of the basis for immunity to influenza in man. Using responses described in infected animals as a guide, none of the recently described cell mediated immune mechanisms are candidates for a primary role in resistance to infection. Most, if not all, however, are undoubtedly involved in recovery from an established infection. For resistance to infection, the major consideration is whether antibody to HA in serum or secretions mediates the resistance. This question was examined in the present report with the aid of sensitive RIP assays for detection of antibody. In three separate studies involving two subtypes of type A influenza and immunization with both live and inactivated vaccine, serum antibody remains the immune mediator most consistently and most strongly related to resistance to infection. Moreover, passive immunization of infants with serum IgG antibody has been recently shown to be associated with protection against influenza. Thus, we believe currently available evidence favors the concept that serum IgG antibody to the influenza virus HA is not only the best correlate but is probably the primary mediator of immunity to influenza. Such a concept of immunity to influenza has also recently been proposed by Potter and Oxford (25). Secretory IgA is capable of neutralizing influenza virus and probably can contribute to resistance. Moreover, the present data suggest that IgG in secretions may have a greater role in resistance than generally attributed to it. An exaggerated antibody response can still be obtained in secretions by repeated topical administration of antigen; in this unusual circumstance, antibody in secretions could assume the primary role of mediating antibody.

Little is known about the source of IgG in secretions or its interrelationships with serum IgG. Differences in occurrence of IgG antibody in secretions may partly account for the prevailing concept that live infection produces better immunity than inactivated vaccines despite comparable serum antibody titers. However, differences in avidity and/or specificity of IgG antibodies induced by different types of antigen presentation could be of equal or greater importance. Assessment of the qualitative differences between antibodies and their relationship to immunity may clarify many of the apparent inconsistencies between serum antibody level and immunity to influenza in man.

REFERENCES

1. Stuart-Harris, C. H., and Schild, G. C., "Influenza," p. 148. Publishing Sciences Group, Inc., Littleton, Massachusetts, (1976).
2. Hobson, D., Curry, R. L., Beare, A. S., and Ward-Gardner, A., *J. Hyg. 70*, 767 (1973).
3. Couch, R. B., *in* Dev. in Biol. Stand. *28*, 295. S. Karger AG, Basel, (1975).
4. Schulman, J. L., *in* "The Influenza Virus and Influenza," p. 373. Academic Press, New York, (1975).
5. Allison, A. C., *Prog. Med. Virol. 18*, 15 (1974).
6. Butchko, G. M., Armstrong, R. B., and Ennis, F. A., *J. Immunol. 121*, 2381 (1978).
7. Leung, K.-N., Ada, G. L., and McKenzie, J. F. C., *J. Exp. Med. 151*, 815 (1980).
8. Stuart-Harris, C. H., and Schild, G. C., "Influenza," p. 52. Publishing Sciences Group, Inc., Littleton, Massachusetts, (1976).
9. Greenberg, S. B., Criswell, B. S., Six, H. R., and Couch, R. B., *J. Immunol. 119*, 2100 (1977).
10. Santoli, D., Trinchieri, G., and Koprowski, H., *J. Immunol. 121*, 532 (1978).
11. Zweerink, H. J., Askonas, B. A., Millican, D., Courtneidge, S. A., and Skehel, J. J., *Eur. J. Immunol. 7*, 630 (1977).
12. Effros, R. B., Bennick, J., and Doherty, P. C., *Cell. Immunol. 36*, 343 (1978).
13. Reiss, C. S., and Schulman, J. L., *J. Immunol. 125*, 2182 (1980).
14. Biddison, W. E., Shaw, S., and Nelson, D. L., *J. Immunol. 122*, 660 (1979).
15. Ennis, F. A., Martin, W. J., and Verbonitz, M. W., *J. Exp. Med. 146*, 893 (1977).
16. Greenberg, S. B., Criswell, B. S., Six, H. R., and Couch, R. B., *Infect. Immun. 20*, 640 (1978).
17. Schulman, J. L., Khakpour, M., and Kilbourne, E. D., *J. Virol. 2*, 778 (1968).
18. Couch, R. B. Kasel, J. A., Gerin, J. L., Schulman, J. L., and Kilbourne, E. D., *J. Infect. Dis. 129*, 411 (1974).
19. Francis, T., Jr., *Science 97*, 229 (1943).
20. Fazekas de St. Groth, S., *Lancet 1*, 1101 (1950).
21. Six, H. R., and Kasel, J. A., *J. Clin. Microbiol. 7*, 165 (1978).
22. Kasel, J. A., Six, H. R., Oborn, C. J., and Dreesman, G. R., *J. Clin. Microbiol. 8*, 171 (1978).
23. Couch, R. B., Douglas, R. G., Jr., Fedson, D. S., and

Kasel, J. A., *J. Infect. Dis. 124*, 473 (1971).
24. Puck, J. M., Glezen, W. P., Frank, A. L., and Six, H. R., *J. Infect. Dis. 142*, 844 (1980).
25. Potter, C. W., and Oxford, J. S., *Brit. Med. Bull. 35*, 69 (1979).

THE SPECIFICITY OF T CELLS FOR INFLUENZA VIRUS HEMAGGLUTININ[1]

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ABSTRACT

The specificity of T cells recognizing influenza virus hemagglutinin (HA) has been studied using functional assays for helper (T_H), suppressor (T_S) and delayed-type hypersensitivity (T_D) T cells. T_H cells raised against one strain of influenza virus cross-react strongly with HA from other viruses of the same subtype, and evidence was also obtained for cross-reactivity between HA subtypes. By using isolated heavy (HA_1) and light (HA_2) chains and cyanogen bromide-derived fragments of A/Memphis/102/72 HA as antigen in each of the assays, determinants recognized by T cells have been assigned to particular regions of the HA molecule. Determinants for T_H cells are carried by the peptides HA_1CN1 and HA_1CN3, and a determinant is also present on HA_2. T_S cells are induced by HA_1CN1 and HA_1CN3, whereas only weak suppression is induced by HA_2. Determinants for T_D cells are carried by HA_1CN1 and HA_2CN1. Thus, not only may some determinants for T cells be different from those recognized by B cells, but also the determinants important in stimulation of T_H, T_S and T_D cells may not be identical.

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INTRODUCTION

T lymphocytes play a central role in the immune response to influenza virus. The humoral immune response to the hemagglutinin (HA) is T cell-dependent, being regulated quantitatively, and probably qualitatively, by both helper T (T_H) cells and suppressor T (T_S) cells. Moreover, cytotoxic T (T_C) cells and T cells mediating delayed-type hypersensitivity (T_D) are probably both important in recovery from influenza virus infection.

Much progress is currently being made towards identifying the serologically-defined antigenic determinants on influenza virus HA (1,2, this volume). In contrast, little is known of the antigenic determinants of HA recognized by T cells. For some non-viral antigens, the finding that receptors on T and B lymphocytes bear similar idiotypes (3,4) suggests that the same range of determinants is recognized by both cell types. Other data however, indicate that T_H cells may have a broader specificity than B cells (5), or that T_H cells, although as specific as B cells, do not recognize an identical range of determinants (6). Considerable evidence exists that determinants reacting with T cells may be less conformation-dependent than determinants reacting with antibody (7) although this may not be true of T_S cells (8). In the case of the lysozymes, T_H cells appear to interact predominantly with a different portion of the molecule to that recognized by serum antibody (9). Furthermore, for the lysozymes (10) and β-galactosidase (11), different regions of the molecule have been found to be important in the induction of T_H cells and T_S cells.

This paper describes preliminary results of our attempts to identify the regions on HA that are recognized by T_H, T_S and T_D cells of BALB/c mice. Using functional assays for each of these T cell types, we have examined the ability of HA-specific T cells to recognize heterologous HA of the same or different subtype, and as a finer probe, to recognize fragments derived from homologous HA by cyanogen bromide (CNBr) cleavage.

METHODS

Viruses. The following strains of influenza virus were used (12): Bel = A/Bellamy/42 (H1N1); Port Chalmers = A/Port Chalmers/73 (H3N2); the "recombinant" viruses $Aichi_H$-Bel_N = A/Aichi/2/68 x A/Bel/42(H3N1), Mem_H-Bel_N =

A/Memphis/102/72 x A/Bel/42(H3N1), Jap_H-Bel_N = A/Jap/305/57 x A/Bel/42(H2N1), $shearwater_H$-Bel_N = A/shearwater/ E.Aust./1/72 x A/Bel/42 (H6N1); and the type B virus B/HK = B/Hong Kong/8/73. Viruses were grown in the allantoic cavity of 10-day embryonated hens' eggs for 2 or 3 days and the allantoic fluid was frozen in aliquots at -70°C and used as the source of live virus. Viruses were concentrated and purified by the method of Laver (13). Purified virus used in culture and for the induction of DTH was inactivated by exposure to UV light (15 watt, 15 cm, 1 min).

HA and fragments of HA. These were kindly prepared by Drs. C.W. Ward and T.A.A. Dopheide. HA was prepared from purified "recombinant" viruses bearing the SDS-sensitive N1 neuraminidase of A/Bel/42, by electrophoresis of SDS-disrupted virus on cellulose acetate blocks (14). Isolated heavy (HA_1) and light (HA_2) chains were separated by guanidine hydrochloride density gradient centrifugation (15). Fragments of Mem HA and HA_1 were prepared by cyanogen bromide (CNBr) cleavage and isolated as previously described (16,17). Fig. 1 shows a schematic representation of the location of the peptide fragments within HA, and the legend gives details of the fragment preparations used in the present study.

Mice. Inbred female BALB/c mice were used.

Preparation of lymphocytes for culture. Methods used for the preparation and culture of spleen cells and for the selective depletion of T or B cells have been described (18). Briefly, for use as a source of unprimed B cells plus macrophages (MØ) in helper assays, spleen cells depleted of T cells were prepared by pretreatment of mice *in vivo* with antithymocyte serum 2 days before sacrifice, followed by treatment of spleen cells *in vitro* with rabbit anti-mouse T cell serum plus complement. For use as a source of T_H cells, spleen cells depleted of B cells were obtained by irradiating the mice (650R) prior to taking the spleens and then passing the spleen cells through nylon wool columns.

Assay of helper T cells. An assay was developed which detects nonspecific help generated by the interaction of influenza virus-primed T cells with purified HA or fragments of HA (19). Mice were immunized intraperitoneally (i.p.) with 200-400 HAU of live influenza virus 4 to 5 weeks before assay for T_H cell activity. Mishell-Dutton-type cultures (20) were set up in two ways. The standard assay used 24-well culture trays, and each well contained 6 x 10^6

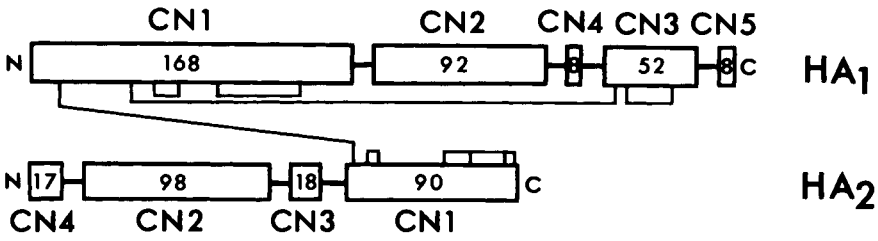

FIGURE 1. Schematic representation of the HA monomer of influenza virus A/Memphis/102/72 and its CNBr cleavage products. The number of amino acid residues in each fragment is shown, and the location of intrachain and interchain disulfide bridges is indicated by solid lines. CNBr cleavage of separated HA_1 yields a mixture of three major fragments in oxidized form. These are HA_1CN1, HA_1CN2 and HA_1CN3 which together are referred to as HA_1Pox; the two octapeptides are lost on dialysis. Cleavage of whole HA gives rise to 3 preparations that can be separated by gel filtration (16,17): P1, which is the sulfide-linked complex of HA_1CN1, HA_1CN3 and HA_2CN1; P2, a mixture of HA_1CN2 and HA_2CN2; and P3, a mixture of the small peptide fragments HA_1CN4, HA_1CN5, HA_2CN3 and HA_2CN4 which can be isolated and purified by high voltage paper electrophoresis. The term HACNBr refers to CNBr-cleaved whole HA which has been dialysed but not subjected to gel filtration. HACNBr contains a mixture of P1 and P2, the small peptides being lost on dialysis. The individual peptides comprising P1 can be obtained by reduction of P1 and subsequent gel filtration followed by controlled reoxidation of the separated fragments. The fragment HA_2CN1 obtained in this way is highly insoluble once it has been lyophilized from formic acid. However, concentration of column fractions containing the fragment by vacuum dialysis, with subsequent dialysis into saline, resulted in a preparation of HA_2CN1 which, although not soluble, could be converted into a homogeneous suspension by sonication. Reproduced from (48), with permission from J. Immunol.

influenza virus-primed or unprimed T cells (prepared as above), 2.5 x 10^6 unprimed B cells + MØ, 2 x 10^6 sheep red blood cells (SRBC) and various doses of purified HA in 0.5 ml medium. For testing the response to fragments of HA, microcultures were set up in 96-well culture trays, and contained one-fifth the number of each cell type used in the standard assay, in a volume of 0.1 ml. Cultures were fed daily with 50 µl or 10 µl, respectively of a concentrated nutrient mixture . After 4 days incubation (37°C, 5% CO_2), cultures were assayed for direct (IgM) anti-SRBC plaque-forming cells (PFC) by the method of Cunningham and Szenberg (21).

Generation and assay of suppressor T cells. Mem_H-Bel_N virus-primed spleen cells were obtained from mice that had been immunized with 200-400 HAU of live virus at least 3 months previously. For the generation of suppressor cells, 1 x 10^7 spleen cells in 1 ml medium were cultured in Marbrook chambers with 1000 HAU of UV-inactivated purified Mem_H-Bel_N virus or 1 µg Mem HA or HA fragments. After 4 days, viable cells were recovered on Isopaque/Ficoll (22), washed, and 10^6 cells were added to fresh, optimally stimulated cultures to assay for their suppressive activity. These second stage cultures contained 1 x 10^7 Mem_H-Bel_N virus-primed spleen cells and 10 HAU of inactivated purified Mem_H-Bel_N virus. Anti-Mem HA antibody was assayed in the culture supernatants after 10 days by using an indirect radioimmunoassay (RIA) in which antibody binding to wells coated with Mem HA is quantitated by subsequent binding of ^{125}I-rabbit anti-mouse IgG. Antibody concentrations are expressed in RIA units whose derivation has been described (18).

Assay of delayed-type hypersensitivity. Mice, which had been pretreated with cyclophosphamide (200 mg/kg body weight, i.p.) 2 days previously, were immunized with 10 µg purified inactivated influenza virus subcutaneously over the right flank. DTH was elicited 6 days later by inoculation of 10 µg purified HA or HA fragments, in 50 µl saline, subcutaneously into the right hind footpad. A group of 5 virus-primed and a group of 5 control (either unprimed or $shearwater_H$-Bel_N virus primed) mice received the same eliciting antigen. The thickness of the right and left hind footpads was measured with calipers 24 hr after inoculation. The level of DTH is expressed as the mean percentage increase in footpad thickness at 24 hr for each group of mice, i.e. the mean of [(R - L)/L)] x 100. Statistical analysis was performed using Student's t-test.

RESULTS

Recognition of HA by Helper T Cells

Helper T cells have been shown to be of two types, one of which provides so called "antigen-specific help", helping B cells specific for determinants carried on the same molecule as stimulated the T_H cell (23,24), and the other providing so-called "nonspecific help", which can help in the response of bystander B cells to an unrelated antigen such as sheep red blood cells (SRBC) (25,26). Both types of T_H cell, however, require antigen for their activation, and there is evidence that both are required in the antibody response to protein antigens (27,28). We chose to use the generation of nonspecific help as an index of T_H cell activation so that the response to fragments of HA could be studied independently of the B cell response to the same fragment. Nonspecific help is assayed as help for the response of primary B cells to SRBC.

Response of T_H cells from Influenza Virus-Primed Mice to HA from Homologous and Heterologous Strains of Virus. Culture of influenza virus-primed T_H cells with homologous purified HA in the presence of unprimed B cells and SRBC led to the generation of significant levels of nonspecific help, as detected by production of anti-SRBC PFC (Fig. 2; see, for example, response of $Aichi_H$-Bel_N primed T_H cells to Aichi HA). Cultures lacking HA gave low background numbers of PFC, as did parallel cultures containing unprimed T_H cells in the presence or absence of added HA.

In order to examine the fine specificity of the T_H cells, the response to heterologous HAs was also tested and the results are summarised in Fig. 2. Mice primed with any of the H3 viruses ($Aichi_H$-Bel_N, Mem_H-Bel_N, Port Chalmers) showed strong cross-reactivity for other HAs of the H3 subtype. In addition, several examples of cross-reactivity for HA of a different subtype were observed. T_H cells from mice primed to any of the H3 viruses or to A/Bel/42(H1) responded to Jap HA (H2). Furthermore, T_H cells from mice primed to Jap_H-Bel_N and, to a lesser extent, A/Bel/42 responded to Mem HA and Port Chalmers HA.

The cross-reactivity observed between subtypes was type A-specific and not due to recognition of common carbohydrate components on the HA of all egg-grown influenza viruses (29,30). T_H cells from mice primed to live egg-grown B/HK

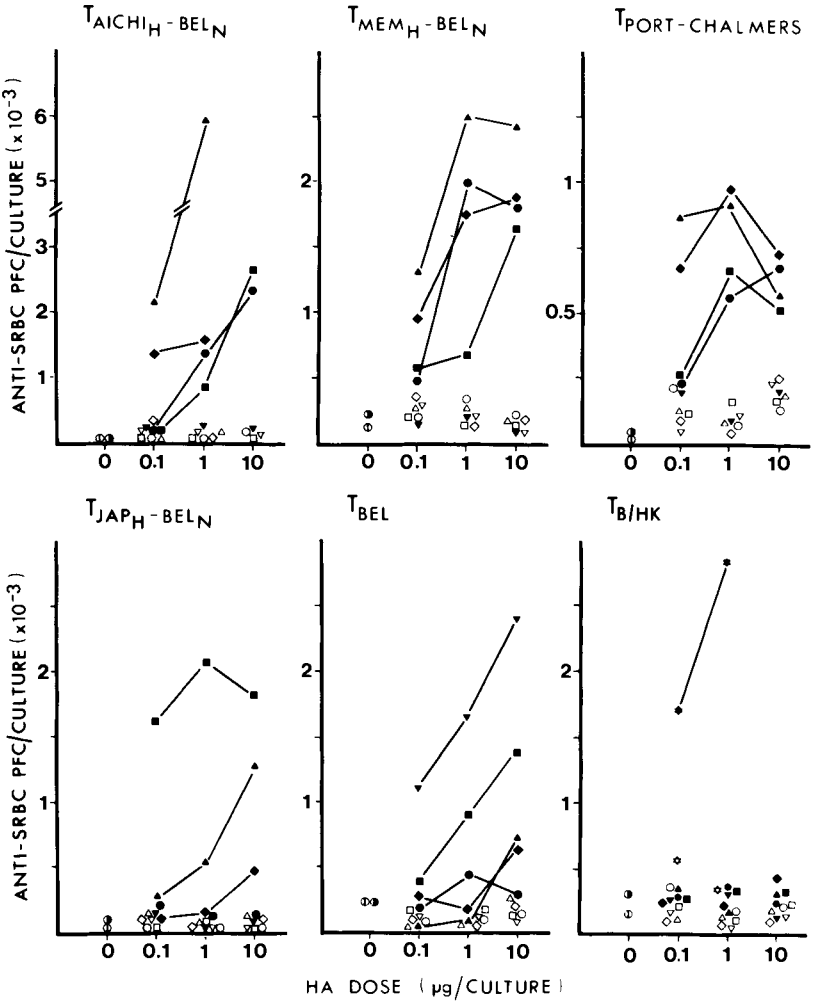

FIGURE 2. Generation of nonspecific help by the interaction of influenza virus-primed T_H cells with HA prepared from homologous and heterologous strains of virus. Cultures contained 6 x 10^6 virus-primed T_H cells (closed symbols) or unprimed T_H cells (open symbols), 2.5 x 10^6 unprimed B cells + MØ, 2 x 10^6 SRBC and various doses of the different HAs: Aichi HA (● ○), Mem HA (▲ △), Port Chalmers HA (◆ ◇), Jap HA (■ □), Bel HA (▼ ▽), or B/HK virus (✱ ✲, tested at 100 and 1000 HAU/culture), or no antigen (◐ ◑), in a total volume of 0.5 ml. Each point represents the mean number of anti-SRBC PFC obtained after 4 days from duplicate or triplicate cultures.

virus failed to respond to any of the purified type A HA preparations while giving a substantial response to inactivated B/HK virus itself (Fig. 2).

Since T_H cells with specificity for matrix protein have been described by other authors (31), we examined the HA preparations for evidence of trace contamination with matrix and other virion proteins by SDS polyacrylamide gel electrophoresis (kindly carried out by Michael Dyall-Smith). Fifty microgram samples of HA were applied to discontinuous gradient (10-20%) Laemmli slab gels which were run under reducing and non-reducing conditions and stained with Coomassie blue. In no case was a band corresponding to matrix protein observed. In the preparations of Jap, Port Chalmers and Bel HA, but not Mem or Aichi HA, a faint band migrating in the position expected for ribonucleoprotein (RNP) was detectable under reducing conditions only. The presence of detectable RNP did not correlate with the ability of the HA preparations to elicit a cross-reactive response, since Bel HA was not cross-reactive in the T_H cell assay whereas Mem HA was. The cross-reactivity observed between subtypes in the T_H cell assay thus appears not to be due to either RNP or matrix protein.

The results indicate that HAs of different subtype may share cross-reactive antigenic determinants recognized by T_H cells. Within a subtype, HAs from different strains are highly cross-reactive with respect to T_H cell recognition.

Response of Mem_H-Bel_N Virus-Primed T_H Cells to Fragments of Mem HA. T_H cells from Mem_H-Bel_N primed mice and unprimed mice were tested in the microassay for reactivity to the following antigens (described in Fig.1 legend): Mem HA, HACNBr, HA_1, HA_2, HA_1Pox, P1, P2, P3, HA_1CN1, HA_1CN3 and HA_2CN1. The results are given in Fig. 3.

In each case the response of unprimed T_H cells was very low. Cleavage of HA with CNBr, or into isolated HA_1, and HA_2 chains, did not result in loss of ability to interact with T_H cells. The fragment HA_1CN1 and the larger fragments containing this region (P1, HA_1, HA_1Pox, HACNBr) were capable of stimulating T_H cells previously primed to virus. Lower but reproducible responses were obtained to HA_1CN3 and P2 (HA_1CN2 + HA_2CN2), whereas P3, the mixture of small peptides (HA_1CN4, HA_1CN5, HA_2CN3, HA_2CN4) did not show activity.

Intact HA_2 also contained one or more determinants for T_H cells. The response to HA_2CN1, however, was poor and not reproducible. The HA_2 determinant may be carried on HA_2CN2, contributing to the P2 response, or may be lost on cleavage of the HA_2 chain.

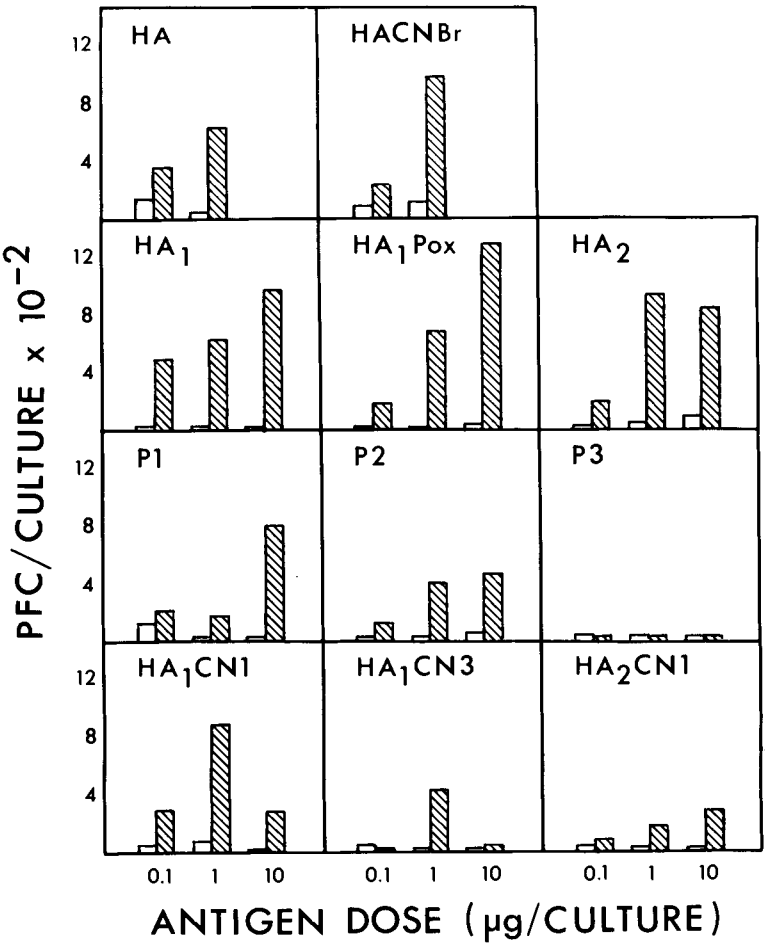

FIGURE 3. Generation of nonspecific help by Mem_H-Bel_N virus-primed T cells in response to Mem HA and fragments of HA. Microcultures contained 1.2 x 10^6 virus-primed T_H cells (hatched bars) or unprimed T_H cells (open bars), 5 x 10^5 unprimed B cells + MØ, 4 x 10^5 SRBC and the indicated doses of influenza antigen in a total volume of 0.1 ml. After 4 days incubation, triplicate cultures were pooled and anti-SRBC PFC enumerated. Values represent mean response per 0.1 ml culture. The PFC response in parallel cultures containing no antigen has been subtracted (138 PFC with virus-primed T cells; 90 PFC with unprimed T cells).

TABLE 1. Generation of Suppressor T Cells by Culture of Mouse Spleen Cells with High Concentrations of Inactivated Influenza Virus[a]

Treatment of precultured cells[b]	Source of 4-day precultured cells				
	None added	Mem_H-Bel_N primed spleen cells cultured with		Unprimed spleen cells cultured with	
		Mem_H-Bel_N virus (1000 HAU)	no antigen	Mem_H-Bel_N virus (1000 HAU)	no antigen
None	23,250[c]±2380	1,090±650(5%)[d]	13,130±1260(57%)	1,480±880(6%)	20,400±8380(88%)
C' alone		630±330(3%)		1,430±250(6%)	
anti-T cell serum + C'		19,050±3890(82%)		20,480±2010(88%)	

[a] 10^6 precultured cells were added to fresh cultures containing 1 x 10^7 Mem_H-Bel_N primed spleen cells and 10 HAU of UV-inactivated purified Mem_H-Bel_N virus. After 10 days incubation, antibody was measured in the culture supernatants by RIA.

[b] 10^6 precultured cells were treated with rabbit anti-mouse T cell serum plus complement, or complement alone, before adding to the second stage cultures.

[c] Anti-Mem HA antibody in RIA units/ml (18). Mean of quadruplicate cultures ± S.E.M.

[d] Percentage of the antibody level obtained in cultures receiving no precultured cells.

Recognition of HA by Suppressor T cells

Induction of T_S Cells by Inactivated Influenza Virus. A strong secondary IgG response to HA can be obtained in vitro in Marbrook-type cultures of influenza virus-primed mouse spleen cells stimulated with UV-inactivated influenza virus (18). Anti-HA antibody present in the culture supernatants after 10 days is measured by RIA. Cultures containing high doses of virus antigen (100-1000 HAU) are found to produce markedly less antibody than cultures containing lower antigen doses (1-10 HAU) (18).

In order to test for the involvement of suppressor cells at high virus concentrations, cultures of Mem_H-Bel_N primed spleen cells containing 1000 HAU of inactivated Mem_H-Bel_N virus were harvested after 4 days. Viable cells were recovered and washed and various numbers transferred into fresh cultures of 1×10^7 Mem_H-Bel_N primed spleen cells containing inactivated Mem_H-Bel_N virus at a dose (10 HAU) optimal for induction of antibody synthesis. In all experiments, cells precultured in the absence of virus antigen were included as controls because of occasional and variable generation of nonspecific suppressor cells by culture of spleen cells in medium alone, as has been documented in the literature (32,33). Addition of 10^6 of the cells precultured with virus suppressed the anti-HA response of the second stage cultures by more than 90% (Table 1). A similar level of suppression was obtained when unprimed spleen cells were precultured with 1000 HAU of virus, indicating the occurrence of a strong primary suppressor response in vitro.

The suppression mediated by cells precultured with virus was abrogated by treatment of the cells with anti-T cell serum plus complement prior to transfer (Table 1). This indicates that virus-induced suppression is T cell-mediated, and eliminates passive carry-over of high concentrations of virus antigen as a possible cause of suppression in the second stage cultures.

Induction of T_S Cells by HA and Fragments of HA. Purified Mem HA, HA_1, HA_2 and the CNBr-derived fragment preparations were tested for their ability to induce suppressor cells which would suppress the anti-HA antibody response in fresh cultures of Mem_H-Bel_N primed spleen cells containing 10 HAU of inactivated Mem_H-Bel_N virus. In the first stage cultures, Mem_H-Bel_N primed spleen cells were incubated with 1 µg of each of the antigen preparations.

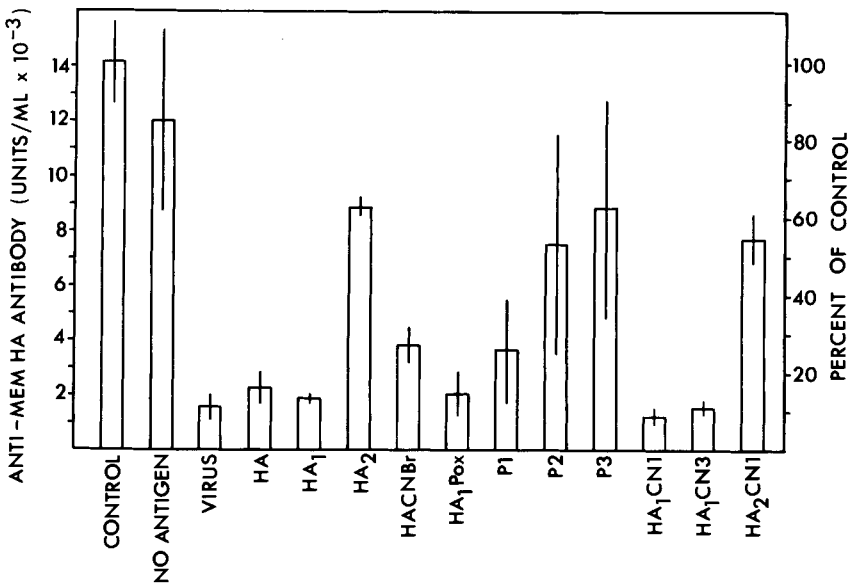

FIGURE 4. Induction of suppressor cells by fragments of Mem HA. Experimental design was the same as in Table 1 except that first stage cultures (1 ml) contained 1 x 10^7 Mem_H-Bel_N primed spleen cells incubated with no antigen, 1000 HAU of inactivated Mem_H-Bel_N virus or 1 µg of each of the HA preparations as indicated. Columns represent mean antibody synthesised in quadruplicate second stage cultures ± S.E.M. "Control" represents second stage cultures receiving no precultured cells.

The results are shown in Fig. 4. Cells precultured with 1 µg Mem HA were almost as suppressive as those precultured with virus. Marked suppressive capacity was also induced by the fragments HA_1CN1 and HA_1CN3, and by other preparations containing these fragments (HA_1, HA_1Pox, HACNBr, P1). The fragment preparations P2 (HA_1CN2 + HA_2CN2), P3 (the mixture of small peptides), HA_2 and HA_2CN1, were much less suppressive.

Recognition of HA by T Cells Mediating Delayed-Type Hypersensitivity

Cyclophosphamide-pretreated mice which had been immunized with 10 µg purified UV-inactivated Mem_H-Bel_N virus

and challenged 6 days later in the footpad with 10 μg purified Mem HA gave a strong DTH reaction at 24 hr (Table 2). Mice which had ben primed with the serologically unrelated type A virus shearwater$_H$-Bel$_N$(H6N1) or with B/HK virus gave intermediate DTH responses to Mem HA which were significantly lower than that of Mem$_H$-Bel$_N$ primed mice, but significantly higher than the response of unprimed mice. In a separate experiment, a similar, intermediate level of DTH was found when Mem$_H$-Bel$_N$ primed mice were challenged with Jap (H2), Bel (H1) or shearwater (H6) HA (data not shown). This partial cross-reactive response was assumed to be directed towards chicken host carbohydrate present on HA.

TABLE 2. DTH Elicited by Influenza HA[a]

Challenge antigen	Immunizing virus			
	None	Mem$_H$-Bel$_N$	Shearwater$_H$-Bel$_N$	B/HK
Mem HA	3.8±1.2	47.8±0.8	17.2±5.5[b]	15.1±3.6[b]

[a] DTH is expressed as the mean percentage increase in footpad thickness ± S.E.M.

[b] Each significantly lower ($p<0.001$) than the response of Mem$_H$-Bel$_N$ primed mice, but significantly higher ($p<0.02$) than the response elicited in unprimed mice.

Response of Virus-Primed T_D Cells to Fragments of HA. Purified Mem HA, HA_1 and HA_2, and each of the CNBr-derived fragment preparations from Mem HA (see Fig. 1) were tested for their ability to elicit DTH in mice primed to either Mem$_H$-Bel$_N$ or shearwater$_H$-Bel$_N$ virus (Fig. 5). Shearwater$_H$-Bel$_N$ primed mice were used in preference to unprimed mice in order to control for DTH responses directed against host carbohydrate.

A significantly higher response in mice primed to the homologous virus compared to the heterologous virus was observed when the eliciting antigen was either HA, HACNBr, HA_1, HA_1Pox, P1, HA_1CN1 or HA_2CN1. It is thus possible to locate determinants for T_D cells on HA_1CN1 and HA_2CN1. The HA_1CN3 preparation elicited a slightly higher response in mice primed to the homologous virus but the difference was not statistically significant. An unexpected finding was the

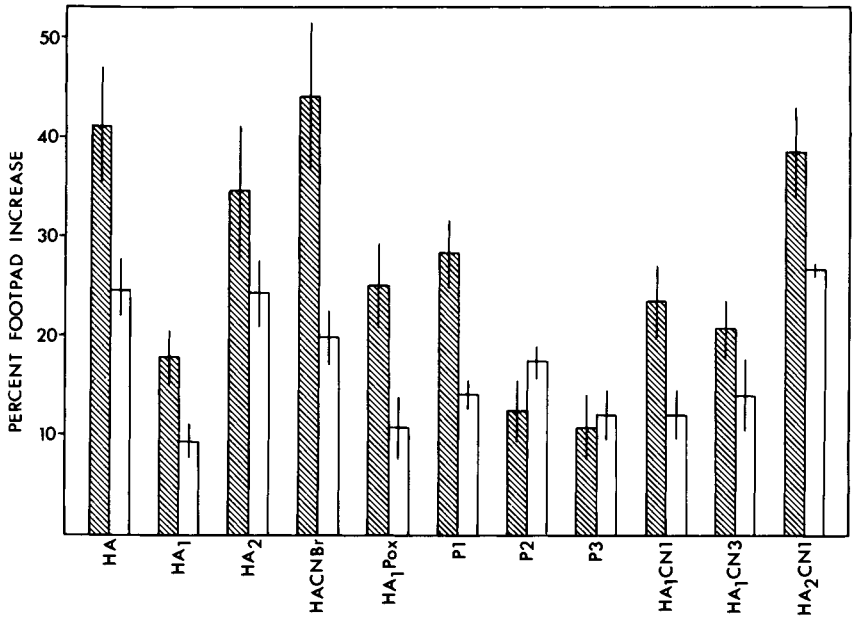

FIGURE 5. DTH response of Mem_H-Bel_N primed mice (hatched bars) and $shearwater_H$-Bel_N primed mice (open bars) to fragments of Mem HA. The difference in the response of the two groups of mice was significant when the challenge antigen was HA ($p<0.05$); HA_1 ($p<0.05$); HACNBr ($p<0.02$); HA_1Pox ($p<0.05$); P1 ($p<0.01$); HA_1CN1 ($p<0.05$); HA_2CN1 ($p<0.05$).

relatively high cross-reactive response obtained in $shearwater_H$-Bel_N primed mice challenged with Mem HA_2 and HA_2CN1 which carry only a minority of the total carbohydrate of HA. This is discussed further below.

DISCUSSION

Our first approach to studying the specificity of T cells recognizing HA was to examine whether T cells primed to one strain of influenza virus could recognize HA from heterologous strains of the same or different subtype. In the case of T_H cells, strong cross-reactivity was found for HAs within a subtype, and some cross-reaction was observed towards HAs of a different subtype. The latter cross-reactivity was type A-specific and not due to a

response of T_H cells to host-derived carbohydrate which is common to egg-grown type A and B influenza viruses (29). If T_H cells with specificity for chicken host carbohydrate exist, they apparently do not become primed in significant numbers when mice are immunized i.p. with a low dose of live virus.

The DTH assay, on the other hand, appears to have a cross-reactive component directed towards host carbohydrate. This may reflect a different priming regime from the helper assay, mice receiving an approximately 10 fold higher dose of purified inactivated virus subcutaneously. The response to carbohydrate may account for only part of the observed response of $shearwater_H$-Bel_N primed mice to Mem HA, however, since Mem HA_2 and HA_2CN1, which each carry only a single oligosaccharide side chain, elicited a higher cross-reactive response than did Mem HA_1, which carries 6 such side chains (34,35). This suggests that Mem HA and shearwater HA may share a polypeptide determinant for T_D cells on HA_2, possibly in the region corresponding to the HA_2CN1 fragment of Mem HA. Further investigation of this possibility requires the use of mouse-grown viruses for priming the mice in order to eliminate the anti-carbohydrate component from the response.

We have been unable to use the same approach with suppressor T cells since culture of spleen cells with HA in vitro induces a strong primary suppressor response. Experiments are in progress to test the specificity of the induced suppressor cells.

The activity of cytotoxic T (T_C) cells directed towards influenza virus-infected target cells has been shown by a number of authors to have a strain-specific component and a component which is broadly cross-reactive towards all type A viruses (36-38). Part of the cross-reactivity may be directed towards matrix protein expressed on the surface of infected target cells (39-42). At least part of the cross-reactivity, however, appears to be directed towards HA (43,44). It is of interest that secondary stimulation of primed spleen cells in vitro with virus-infected cells enhances the activity of both the cross-reactive and the strain-specific T_C populations, whereas stimulation with purified HA selectively enhances the strain-specific T_C population (38,45,46). Optimal presentation of the cross-reactive HA determinants to T_C cells, and perhaps to other T cell types, may thus require correct orientation of HA within the membrane of a virion or infected cell.

From the results of the studies with fragments of HA it is possible to locate the determinants recognized by T cells of BALB/c mice on particular cyanogen bromide-derived

fragments of A/Memphis/102/72 HA as follows:

	T_H	T_S	T_D
HA_1	+	+	+
HA_1CN1	+	+	+
CN2	?	-	-
CN3	+	+	?
CN4	-	-	-
CN5	-	-	-
HA_2	+	-	+
HA_2CN1	-	-	+
CN2	?	-	-
CN3	-	-	-
CN4	-	-	-

The failure of a particular fragment to display antigenic activity does not necessarily imply that that region is inactive when present in the intact molecule. Loss of antigenicity of a determinant for T cells could occur on fragmentation if (i) the determinant spans the cleavage site of CNBr; (ii) the fragment fails to be adequately presented by macrophages (in the case of T_H and T_D determinants); (iii) the conformation of the determinant is important and is lost. In many antigen systems, T cell determinants appear to be less conformation-dependent than B cell determinants (7). However, at least for suppressor T cells (8), and perhaps for other T cell types, conformation-dependent determinants may exist.

In comparing the reactivity of different fragments for each of T_H, T_S and T_D cells and for B cells it is apparent that at least some T cell determinants may be different from those recognized by B cells. Furthermore, the determinants important in stimulation of T_H, T_S, and T_D cells may not be identical. T cell determinants are present on both the HA_1 and HA_2 chains of the molecule. Within HA_1, the large fragment HA_1CN1 is the most antigenic for T cells, having determinants recognized by virus-primed T_H and T_D cells and capable of inducing T_S cells. HA_1CN1 is also the major CNBr-derived fragment of HA capable of binding antiviral antibody, and antibody elicited by HA_1CN1 can inhibit viral hemagglutination (47).

HA_1CN3 stimulates a low but reproducible response in virus-primed T_H cells and can induce T_S cells. This fragment does not bind antiviral antibody however, although antibody raised to isolated HA will bind HA_1CN3 (47).

The HA_2 chain gives strong stimulation of virus-primed T_H cells and T_D cells, the latter also being responsive to HA_2CN1. HA_2 did not induce strong suppression. A determinant for antibody has been identified on HA_2CN1 but elicits only a minor proportion of the total antiviral antibody (48). The major role of the light chain in antigenicity may therefore be in the stimulation of T_H and T_D cells rather than B cells.

Whether there is any identity of determinants for T and B cells on HA is unknown. Now that synthetic HA peptides representing the antigenic binding sites for antibody are becoming available (Jackson et al., this volume), it will be of great interest to test these in the T cell assays to determine whether the determinants for T and B cells are identical, overlapping or distinct.

ACKNOWLEDGMENTS

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REFERENCES

1. Laver, G., and Air, G. (1980) (eds.) "Structure and Variation in Influenza Virus". Elsevier North Holland, New York.
2. Wiley, D.C., Wilson, I.A., and Skehel, J.J. (1981) Nature 289, 373.
3. Binz, H., and Wigzell, H. (1975) J. Exp. Med. 142, 197.
4. Eichmann, K., and Rajewsky, K. (1975) Eur. J. Immunol. 5, 661.
5. Hoffmann, M., and Kappler, J.W. (1973) J. Exp. Med. 137, 721.
6. Isac, R., and Mozes, E. (1977) J. Immunol. 118, 584.
7. Arnon, R. and Geiger, B. (1977) in "Immunochemistry: An Advanced Textbook", (L.E. Glynn and M.W. Steward, eds.) John Wiley & Sons, Chichester, p. 307.
8. Endres, R.O., and Grey, H.M. (1980) J. Immunol. 125, 1515.
9. Maizels, R.M., Clarke, J.A., Harvey, M.A., Miller, A. and Sercarz, E.E. (1980) Eur. J. Immunol. 10, 509.

10. Adorini, L., Harvey, M.A., Miller, A., and Sercarz, E.E. (1979) J. Exp. Med. 150, 293.
11. Turkin, D., and Sercarz, E.E. (1977) Proc. Natl. Acad. Sci. USA 74, 3984.
12. Memorandum in Bull. Wld. Hlth. Org. (1980) 58, 585.
13. Laver, W.G. (1969) in "Fundamental Techniques in Virology", (K. Habel and N.P. Salzman, eds.) Academic Press, New York, p.82.
14. Laver, W.G. (1964) J. Mol. Biol. 9, 109.
15. Laver, W.G. (1971) Virology 45, 275.
16. Jackson, D.C., Russell, R.J., Ward, C.W., and Dopheide, T.A. (1978) Virology 89, 199.
17. Jackson, D.C., Dopheide, T.A., Russell, R.J., White, D.O. and Ward, C.W. (1979) Virology 93, 458.
18. Anders, E.M., Peppard, P.M., Burns, W.H., and White, D.O. (1979) J. Immunol. 123, 1356.
19. Anders, E.M., Katz, J.M., Brown, L.E., Jackson, D.C., and White, D.O. (1981) J. Immunol. Submitted for publication.
20. Mishell, R.I., and Dutton, R.W. (1967) J. Exp. Med. 126, 423.
21. Cunningham, A.J., and Szenberg, A. (1968) Immunology 14, 599.
22. Davidson, W.F., and Parish, C.R. (1975) J. Immunol. Methods 7, 291.
23. Feldmann, M., and Basten, A. (1972) Nature New Biol. 237, 13.
24. Taussig, M.J. (1974) Nature 248, 234.
25. Schimpl, A., and Wecker, E. (1972) Nature New Biol. 237, 15.
26. Marrack, P.C., and Kappler, J.W. (1975) J. Immunol. 114, 1116.
27. Keller, D.M., Swierkosz, J.E., Marrack, P., and Kappler, J.W. (1980) J. Immunol. 124, 1350.
28. Tada, T., Takemori, T., Okumura, K., Nonaka, M., and Tokuhisa, T. (1978) J. Exp. Med. 147, 446.
29. Harboe, A. (1963) Acta Pathol. Microbiol. Scand. 57, 317.
30. Jackson, D.C., Brown, L.E., and White, D.O. (1981) J. Gen. Virol. 52, 163.
31. Russell, S.M., and Liew, F.Y. (1979) Nature 280, 147.
32. Burns, F.D., Marrack, P.C., Kappler, J.W., and Janeway, C.A. (1975) J. Immunol. 114, 1345.
33. Schreier, M.H., and Lefkovits, I. (1979) Immunology 36, 743.
34. Ward, C.W., and Dopheide, T.A.A. (1980) Virology 103, 37.

35. Dopheide, T.A., and Ward, C.W. (1980) in "Structure and Variation in Influenza Virus" (G. Laver and G. Air, eds.) Elsevier North Holland, New York, p.21.
36. Effros, R.B., Doherty, P.C., Gerhard, W., and Bennink, J. (1977) J. Exp. Med. 145, 557.
37. Zweerink, H.J., Courtneidge, S.A., Skehel, J.J., Crumpton, M.J., and Askonas, B.A. (1977) Nature 267, 354.
38. Braciale, T.J. (1977) Cell. Immunol. 33, 423.
39. Braciale, T.J. (1977) J. Exp. Med. 146, 673.
40. Biddison, W.E., Doherty, P.C., and Webster, R.G. (1977) J. Exp. Med. 146, 690.
41. Ada, G.L., and Yap, K.L. (1977) Immunochemistry 14, 643.
42. Reiss, C.S., and Schulman, J.L. (1980) Infect. Immun. 29, 719.
43. Askonas, B.A. and Webster, R.G. (1980) Eur. J. Immunol. 10, 151.
44. Koszinowski, U.H., Allen, H., Gething, M.J., Waterfield, M.D. and Klenk, H.D. (1980) J. Exp. Med. 151, 945.
45. Zweerink, H.J., Askonas, B.A., Millican, D., Courtneidge, S.A., and Skehel, J.J. (1977) Eur. J. Immunol. 7, 630.
46. Braciale, T.J. (1979) J. Exp. Med. 149, 856.
47. Jackson, D.C., Brown, L.E., White, D.O., Dopheide, T.A.A., and Ward, C.W. (1979) J. Immunol. 123, 2610.
48. Brown, L.E., Dopheide, T.A.A., Ward, C.W., White, D.O., and Jackson, D.C. (1980) J. Immunol. 125, 1583.

IMMUNE RESPONSES TO INFLUENZA VIRUS IN GUINEA PIGS, MICE AND HAMSTERS

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ABSTRACT

Human influenza virus PR/8/34, was inoculated into the lungs of guinea pigs, mice and hamsters. Whereas .026 hemagglutinating units (HAU) of the virus was lethal to mice, 256 HAU were tolerated by guinea pigs and hamsters. Guinea pigs remained clinically free of disease. Hamsters became ill but survived. Morphologic studies of the lungs of hamsters and mice with influenza infection demonstrated similarities to histologic changes seen in lungs from humans with influenza pneumonia. The kinetics of both cell mediated (thymus derived lymphocyte, B-cell) responses were studied in a variety of lymphoid tissue. T cell responses (^{3}H-TdR incorporation) were widely disseminated throughout the animals' lymphoid tissue as early as one week. Antibody forming cell responses (Jerne plaque) appeared restricted to the draining lymph nodes (hilar). In the hamster, the quality of the T-cell response varied with the strain of hamster used in the experiment. The serum antibody (hemagglutination inhibition) and antibody forming cell response in the guinea pig and hamster appeared to be dependent on the viability of the virus inoculum. Inactivated virus was incapable of stimulating an antibody forming cell response, was markedly less capable than live virus for stimulating serum antibody, but was adequate for stimulating T cells to proliferate. These

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studies demonstrate that the hamster and the guinea pig are useful for the study of immune responses to antigens inoculated into the lungs as well as providing a model for the study of influenza pneumonia.

INTRODUCTION

The respiratory tract is exposed continuously to immunogenic and infectious agents. The lower respiratory tract generally is protected by the defense mechanism of the upper respiratory tract. This protection, however, is not always complete and the lower respiratory tract (lung) may have unique mechanisms for protection (1). A primary area of investigation in our laboratory is to evaluate the defense mechanisms related to the lung.

The purpose of the present studies was to develop an experimental model of influenza pneumonia in laboratory animals and to study the immune response to infectious and inactivated influenza virus. In this manuscript we present data from these studies and compare our observations on the development of influenza pneumonia in three laboratory species (mouse, guinea pig and hamster) and compare the histologic changes induced in their lungs by the virus infection to those changes present in human lungs with influenza pneumonia.

RESULTS

Pathology Associated with Influenza Pneumonia.

In order to induce an influenza pneumonia, mice, guinea pigs and hamsters were anesthetized and inoculated intratracheally (IT) with 0.02, 25, or 256 hemagglutinating units (HAU) of PR/8/34 influenza virus in a volume of 0.05 ml (mouse) or 0.1 ml (guinea pig, hamster) phosphate buffered saline. Intratracheal inoculations were performed as described previously in mice (2) and hamsters (3). In the guinea pig, IT inoculations were accomplished by injecting the inoculum through a 1 ml tuberculin syringe (23 ga. needle), into the lung via an exposed trachea. The wound was surgically clamped and guinea pigs positioned for maximum drainage of the inoculum into the lung. Virus (PR/8/34, HINI) was purchased from American type Culture Collection (Rockville, MD) and was grown in embryonated chicken eggs by standard procedures (4) or was a sucrose gradient purified virus concentrate (a generous gift from M. Phelan and F.

Ennis, M.D., of the Virology Department, Bureau of Biologics). We observed that as little as 0.02 HAU of influenza virus was lethal to mice. They became ill, developed ruffled fur and "chattered" characteristically. Mice died 3-7 days post inoculation. Guinea pigs however remained clinically free of disease when inoculated with 256 HAU of influenza flu virus. The hamsters became ill but survived even after inoculation of 256 HAU PR/8/34. However, histologic examination of changes in the lung of all three animals demonstrated some similarities to histologic changes seen in lungs of humans with influenza pneumonia.

Histologic changes in lungs from a human case available to us who died from influenza pnemonia 3 days after infection showed mononuclear infiltrates in alveolar septal walls as well as increased numbers of mononuclear cells in alveolar spaces. Hyaline membrane, characteristic of human influenza, were prominent in sections that were studied. Bronchiolar epithelial necrosis occurs with influenza pneumonia but this finding was not prominent in the human specimen studied because of autolysis resulting from a delay in autopsy.

As in humans, mice dying from influenza pneumonia at 3 to 7 days demonstrated increased numbers of mononuclear cells

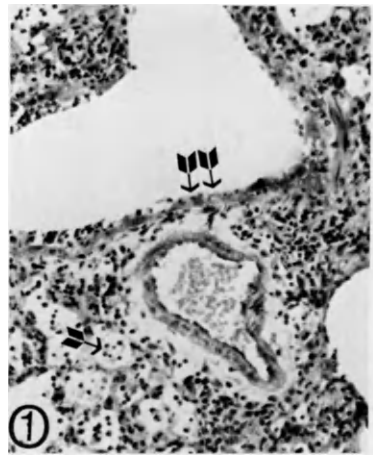

Figure 1. MHA hamster lung 5 days after the inoculation of 256 HAU of influenza virus. There is destruction of bronchiolar epithelium (double arrow), mononuclear cells and exudate in the alveolar spaces (arrow) and thickening of alveolar septal walls by mononuclear infiltrates.

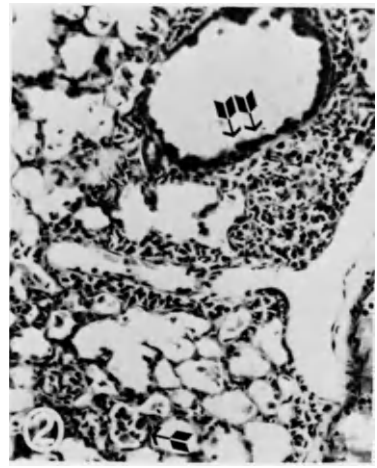

Figure 2. Guinea pig lung 5 days after intratracheal inoculation of 256 HAU of influenza virus. There are peribronchiolar and perivascular mononuclear cell infiltrates. Mononuclear cells hve accumulated in alveolar spaces (arrow) and also infiltrated the alveolar septal walls. There has been no destruction of the bronchiolar epithelium (double arrow).

in the alveolar septal walls and spaces and hyaline membranes. Destruction of the bronchiolar epithelium was prominent.

Hamsters and guinea pigs tolerated 256 HAU of virus. Hamsters demonstrated the greatest infiltration of mononuclear cells in alveolar septal walls and spaces of all three animals studied. Bronchiolar epithelial necrosis was prominent at 5 days (Fig. 1) but by 7 days epithelial regeneration with hyperplasia was evident (not shown). Histologic changes seen in lungs of guinea pigs included mononuclear infiltrates around small vessels and bronchioles as well as in the alveolar septal walls and spaces (Fig. 2). There was no evidence of necrosis of the bronchiolar epithelium. It has previously been reported that inoculation of influenza into the nostrils of guinea pigs failed to result in necrosis of respiratory epithelium although viral replication occurred (5).

We concluded from our histologic studies that deposition of influenza virus in the lung gave a productive infection in all 3 species. All demonstrated histologic changes similar to those changes seen in human influenza disease except in

the case of the guinea pig where bronchial epithelial necrosis was not detected. We next studied both cell mediated and antibody responses in various lymphoid tissues in the guinea pig and hamster infected intratracheally with influenza virus.

Immune Responses of Guinea Pig to PR/8/34.

When 256 HAU of live or heat-inactivated (HI) (56°C-1 h) virus was inoculated into the lung via the trachea and lymphoid tissue was removed on day 5, 7, or 14, a specific incorporation of ^{3}H-TdR could be detected when cells were cultured in the presence of PR/8/34 virus (65 HAU/ml) (Fig. 3). A stimulation ratio above 2 was considered positive. As early as 5 days after infection specific proliferative responses were detected in some animals both locally in lung tissue and hilar lymph nodes (HLN) and n systemic lymphoid tissue including spleen and peripheral lymph nodes (PLN) (Fig. 3). By two weeks proliferative responses were more consistently demonstrated. This widespread dissemination of T cell responses is consistent with the known traffic of specific T cells following their induction in organized lymphoid tissue (6).

When the proliferation response was compared in animals who received live virus with the response of animals who

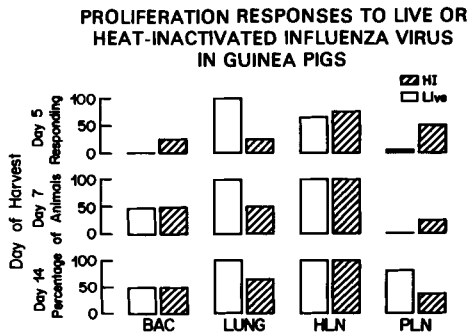

Figure 3. Guinea pigs were inoculated with live or heat inactivated (HI) influenza virus PR/8/34 via the trachea. Lymphoid tissue was harvested on indicated days and cultured with or without virus. ^{3}H-thymidine incorporation was considered specific when the stimulation index (SI) was ≧2. (SI = CPM experimental/CPM control). Tissues assayed were bronchoalveolar cells, (BAC) obtained by bronchiolar lavage; lung cells were removed from lung by mincing and dissociation after lung lavage; hilar lymph nodes, (HLN); and peripheral lymph nodes, (PLN).

TABLE 1: Serum Antibody Titer (Hemagglutination Inhibition)[a]

	Influenza virus[b]	7 days	14 days
Live:	High	1:80	1:1280
	Low	1:640	1:1280
H.I.:	High	neg	1:10
	Low	neg	neg

[a] Hemagglutination inhibition - performed according to Dowdle (7)

[b] Influenza virus was inoculated IT into guinea pigs as live or heat-inactivated (HI) particles in a high (256 HAU) or low (25 HAU) dose on day 0. On day 7 or 14 post inoculation blood was collected and serum prepared.

received heat inactivated virus the following two observations were made: 1) More animals demonstrated virus specific T cells in their lungs if the virus was infectious. 2) In contrast, animals responding to HI virus demonstrated specific T cells in their peripheral lymphoid tissue earlier than animals inoculated with infectious virus. This suggested to us that specific T cells may be recruited or trapped at the site of infection.

The difference in response to infectious and inactivated virus was even more obvious when the serum antibody response was evaluated (Table 1). An antibody titer was detected in the serum by day 7 and 14 if the intratracheal inoculum contained live virus. On the other hand if the virus was heat inactivated, no significant serum antibody titer was detected.

Immune Responses to PR/8/34 in the hamster.

Immune responses in the hamster were studied in response to infectious virus. One week after intratracheal immunization of infectious influenza virus, specific T cell proliferation was seen throughout the lymphoid tissue in some of the animals. The distribution of this response varied according to the strain of hamster inoculated. The response appeared more often in the HLN of the MHA hamsters while the

TABLE 2: Development of Antibody Forming Cells (AFC) to Influenza Virus[a] in Hamsters

Day Harvest	Number Animals[c]	Direct AFC Per Organ ± SEM x 10^{-3} Hilar	Spleen
4	(3)	88.6 ± 31.8	0
7	(9)	48.6 ± 11.5	15.6 ± 5.5
14	(9)	1.6 ± 0.5	10.0 ± 2.4
		Indirect AFC Per Organ ± SEM x 10^{-3}	
		Hilar	**Spleen**
7	(4)	110.0 ± 36	0
14	(7)	1.0 ± 0.4	20.0 ± 5.6

[a] Animals inoculated IT with 0.1 ml of virus in allantoic fluid or 0.1 ml of concentrated virus diluted in phosphate buffered saline to contain 256 HAU of influenza PR/8/34.
[b] SEM, Standard error of the mean.
[c] Results are pooled for three strains of hamsters: MHA, LHC, and LSH.

response was demonstrated in the spleens of 100% of the LHC hamsters. Too few animals were examined to correlate what effects this pattern of response might have on the susceptibility of each strain to the influenza virus disease.

The development of specific antibody forming cell (AFC) responses to influenza virus was studied in the hamster by using a modified Jerne plaque assay (8). Virus was attached to sheep erythrocytes as described previously (9). All three hamster strains studied (MHA, LSH, LHC) responded similarly. At day 4, the majority of the AFC response was detected in lymph nodes draining the lung (hilar). The response was not observed in the peripheral (axillary, brachial, inquinal), or mesenteric lymph nodes. As the response diminshed in the HLN AFC could be detected in the spleen. This observation was true for both direct and indirect AFC. These data suggest that the AFC that initially developed in response to IT inoculation of influenza virus originated in the lymph nodes draining the lung and later appeared in the spleen. The development of AFC in the spleen could be a result of lymphocyte traffic or antigenic drainage to the central lymphoid organ. Unlike T cell responses, the B-cell responses as

measured by AFC were confined to the HLN and spleen and not detected in peripheral or mesenteric lymph nodes (Table 2).

In the few hamsters that were inoculated with heat inactivated virus we were unable to detect an AFC response. This observation correlates with our previous observation in the guinea pig that live virus was better than Hl virus in inducing serum antibody titers.

DISCUSSION

This study has demonstrated the use of three laboratory species (mouse, guinea pig and hamster) as models for studying influenza pneumonia. Our data are consistent with that of others that have indicated that instillation of influenza virus into the lungs of virgin mice results in a disease lethal (10). Most studies in which mice survive influenza inoculation have administered the virus intranasally.

Since the guinea pig and hamster did not die from the influenza infection they are excellent models for studying immune responses related to the lung. The guinea pig is particularly suited for the study of T lymphocyte responses and may be an important model for studying hypersensitivity resulting from cell mediated immunity in the lungs.

We believe that the hamster is an excellent model for studying influenza disease as well as immune responses related to influenza infection. We arrived at this conclusion because of the following observations:

1) The disease produced in hamsters by IT inoculation of influenza PR/8/34 was more similar to human influenza disease than the devastating disease produced in mice, or the mild syndrome observed in the guinea pig, i.e., the hamster becomes ill but recovers.

2) Both T and B lymphocyte responses to influenza can be readily studied in the hamster.

3) The hamster carries a low endogenous bacterial and viral load (11).

In our studies we observed that T-lymphocyte proliferation responses disseminated rapidly throughout the lymphoid tissue and development of these responses was

not dependent on the ability of the virus to infect the animal. The distribution of the T cell responses was, however, dependent on development of infection in the animal. Infection in the lung appeared to trap or recruit more effectively specific T cells to that area.

We observed that the B-lymphocyte (AFC) responses were confined to the lymphoid tissue draining the lung (hilar, later spleen). This suggests to us that the B-lymphocytes in general do not recirculate but are confined to the organs containing the antigen. In our previous model, using non-infectious antigenic particles (sheep erythrocytes) (3), the AFC response was primarily in the hilar lymph nodes unless a large dose was given. Then the splenic response was observed at the same time as the hilar response. In contrast the infectious particles induced the development of AFC in the hilar lymph nodes first and then approximately 3 days later AFC were observed in the spleen. This suggests that 1) the virus was able to reach the spleen but not other peripheral lymphoid tissue and/or 2) there was traffic of AFC precursors from HLN to spleen. Studies are in progress to determine if infectious virus reaches the spleen. It is clear, however, that the distribution of the AFC responses is different depending on the infectivity of the antigen.

The development of antibody responses in both the guinea pig and hamster model was dependent on the ability of the virus to infect the animal. The reason for this observation may be dependent on the amount of antigen ultimately perceived in the lung and/or the mechanisms of clearance of the two particles. The infectious virus replicates in bronchiolar epithelial cells and therefore increases the amount of antigen in the lung. In addition, inactivated virus may be more efficiently removed from the lung so that the amount of antigen presented to the immune apparatus of the lung is reduced. A corollary to this hypothesis is that more antigen is necessary to stimulate the production of antibody than the proliferation of T cells.

In summary, we observed that both the local and systemic immune systems are induced during a local pulmonary infection with influenza. Because the data demonstrate that the infectious virus affects the distribution of the specific T cell response, the appearance of AFC in the hilar lymph nodes (hamster) and the quantity

of serum antibody we conclude that infectious virus produces a more effective immune response. These data support the hypothesis that influenza virus immunity might be induced best by local administration of an attenuated virus.

ACKNOWLEDGEMENTS

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BIBLIOGRAPHY

1. Kaltreider, H.B. (1976). Am. Rev. Resp. Dis. 113, 347.
2. Stein-Streilein, J. and Hart, D. (1976). Infect. and Immun. 30, 391.
3. Stein-Streilein, J., Gross, G.W., and Hart, D.A. (1979). Infect. Immun. 24, 145.
4. Lenett, E.H. et al., ed. (1974). Manual of Clinical Microbiology. American Society for Microbiology, Washington, 678.
5. Stuart-Harris, C.H. (1937. Brit. J. Exp. Path. 18, 485.
6. Ford, W.L. (1975). Prog. Allergy 19, 1.
7. Dowdle, W.R. (1980). in Manual of Clinical Immunology, Rosen and Friedman, H. eds., American Society for Microbiology, Washington, D.C., 634.
8. Stein-Streilein, J. and Hart, D. (1979). Cell. Immunol. 45, 241.
9. McLaren, Colin, Grubbs, G.E. and Ennis, F.A. (1978). J. Clin. Micro. 8, 438.
10. Yetter, R.A., Lehrer, S., Ramphall and Small, P.A. (1980). Infect. and Immun. 29, 654.
11. Toolan, H. (1978). Fed. Proc. 37, 2065.

Recovery from a Viral Respiratory Tract Infection: III. Specificity of Protection Conferred by Immune Spleen Cells Stimulated In Vitro

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ABSTRACT

Nude mice failed to clear influenza virus from their lungs, and eventually died from pneumonia. In this report we describe the pulmonary pathology seen in nude and BALB/c immunocompetent mice infected with mouse adapted A/Port Chalmers/1/73 (H3N2) virus. Experiments were performed in an attempt to alter the course of influenza pneumonia by passive transfer of donor immune spleen cells to infected nude mice. As we reported earlier (1,2), immune spleen cells which are restimulated in vitro have enhanced cytotoxic T cell activity and when transferred to infected nude mice effect recovery. On the contrary, immune spleen cells which are not restimulated prior to transfer augment the antibody response of recipient mice, but do not effect recovery. We now describe the specificity of this protection afforded by these immune spleen cells following secondary in vitro stimulation. These spleen cells were highly protective against lethal challenge by homologous and heterologous virus strains. The strain specific protection was highest but very significant cross subtype protection was observed. This in vivo protection reflected the cross subtype cytotoxic activity noted in target cells following in vitro stimulation prior to transfer of these immune spleen cells to recipient mice. The cross reactive protection was type A specific, since immune spleen cells stimulated by type B virus did not protect.

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INTRODUCTION

In previous reports we described the detection of virus specific, H-2 restricted cytotoxic T cells in the lungs of immunocompetent mice (BALB/c) with influenza pneumonia (3), and compared the pathology in the lung, clearance of infectious virus and viral antigen and survival to influenza infection in T cell deficient mice (1). The nude mice had a later mean day of death than immunocompetent mice, but eventually more nude mice died. Those results indicated that earlier deaths were observed in immunocompetent mice, so immunopathology may contribute to mortality from influenza pneumonia. Eventually, however, nude mice failed to clear virus from their lungs, pathology progressed and more nude mice died. We then analyzed the effect of passive transfer of immune spleen cells to nude mice with influenza pneumonia (2). We found that transfer of spleen cells from BALB/c mice infected 8 days earlier to nude mice on day 2 after infection resulted in some decrease in pulmonary virus titers and increase in antibody response compared to nude mice receiving spleen cells from control mice. The decrease in virus titer was significant but the nude mice continued to have virus in their lungs beyond the time immunocompetent mice cleared the virus. We then analyzed the effect of passive transfer of immune spleen cells, obtained from mice infected 30 days earlier. These spleen cells were then restimulated with virus *in vitro* for five days before transfer or cultured without virus antigen for five days prior to transfer. The immune spleen cells which were secondarily stimulated became highly cytotoxic *in vitro* but the non-stimulated immune cells did not. When transferred to mice with influenza pneumonia, the secondarily stimulated spleen cells were highly effective in the recovery from pneumonia but did not elevate the antibody levels of the recipient mice. On the contrary, the immune spleen cells which were not restimulated markedly enhanced the antibody responses of the nude mice, but did not effect recovery from pneumonia. In the present report we describe the results of experiments designed to define the antigenic specificity of protection mediated by the secondarily stimulated immune spleen cells.

METHODS

Please refer to references 1-5 for detailed descriptions of the methods used in these experiments.

RESULTS

Figures 1 and 2 demonstrate the typical progression in pulmonary pathology observed in BALB/c, and nude mice given $10^{3.5}$ egg infectious $dose_{50}$, which is about 0.1 lethal $dose_{50}$ in the BALB/c mice, of mouse adapted A/Port Chalmers/1/73 (H3N2) virus. Similar results are seen with other influenza A and B viruses.

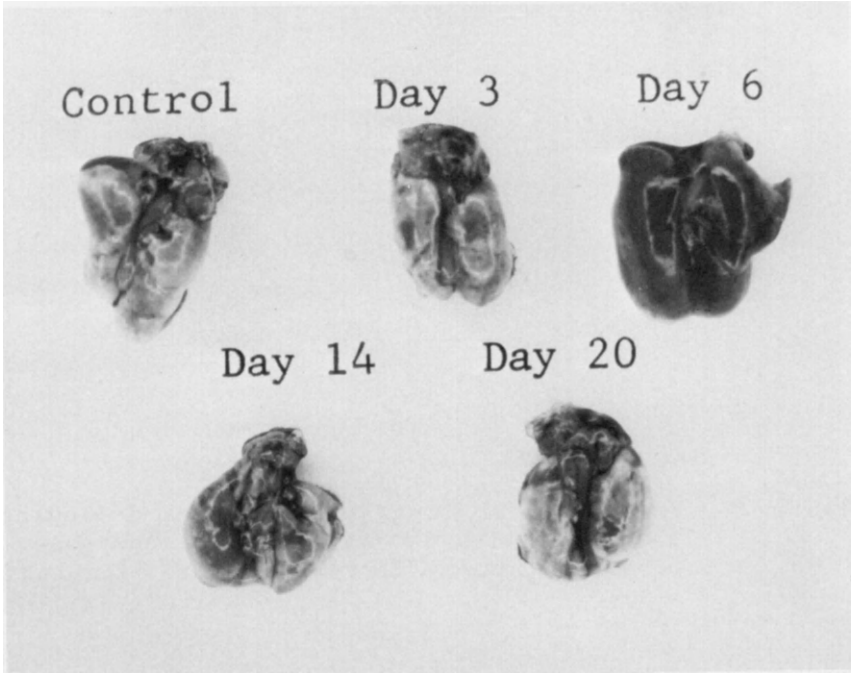

Figure 1. Photograph of lungs of BALB/c mice following infection with $10^{3.5}$ egg infectious $dose_{50}$ of mouse adapted A/Port Chalmers/1/73 influenza virus.

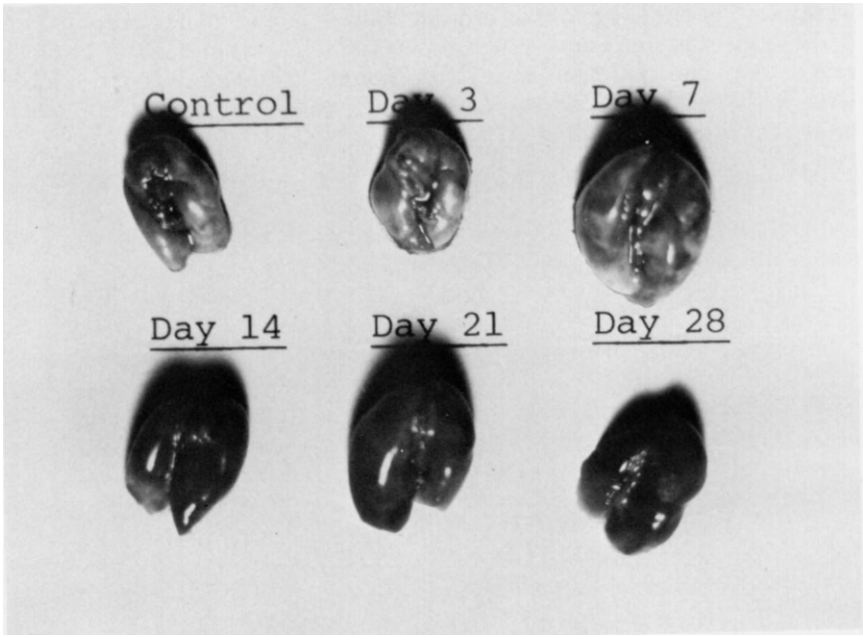

Figure 2. Photograph of lungs of nude mice following infection with $10^{3.5}$ egg infectious dose$_{50}$ of mouse adapted A/Port Chalmers/1/73 influenza virus.

Earlier experiments demonstrated that protection could be transferred to nude mice if they received secondarily stimulated immune spleen cells, which were highly cytotoxic, but not if they had received immune spleen cells which were not restimulated *in vitro* prior to transfer (3). We compared the host responses in nude mice with influenza pneumonia following transfer of control spleen cells, immune spleen cells, and immune spleen cells which were restimulated prior to transfer. Figure 3 illustrates the results of a typical experiment. Nude mice 14 days after infection had marked pulmonary pathology, high pulmonary virus titers and much virus antigen in their lungs. This was not affected by transfer of control spleen cells and only somewhat decreased by transfer of immune spleen cells given 24 hours after infection. Immune spleen cells which were restimulated and

became cytotoxic in vitro, however, were associated with a marked reduction in pulmonary pathology, and the clearance of infectious virus and viral antigens from the lungs. The recovery was accomplished following transfer of these cells which did not increase the negligible antibody response observed in nude mice, and recovery was not enhanced in recipients of immune spleen cells, which had markedly enhanced the antibody response.

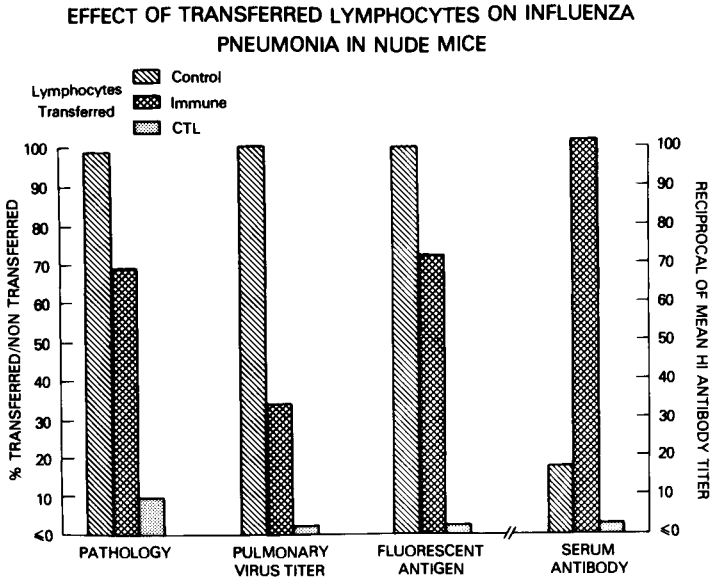

Figure 3. A comparison of the effect of transferred lymphocytes on pulmonary pathology, pulmonary virus titer and expression of fluorescent antigen in pulmonary tissue. These comparisons are expressed as the percentage of infection in nude mice infected 14 days previously with 5 lethal dose$_{50}$ of A/Port Chalmers influenza virus and then receiving 50 x 10^6 lymphocytes one day later, divided by effects on the lung observed in infected nude mice not receiving lymphocytes. The reciprocal of mean hemagglutination-inhibiting antibody titers were measured from day 14 serum.

We then studied the specificity of the protection afforded by these secondarily stimulated immune spleen cells. Donor mice were infected intranasally in the usual way (1-5) with A/Port Chalmers/1/73, A/PR/8/34 or B/HK/5/72 virus strains and their spleen cells were removed 30 days after infection, when cytotoxic T cell activity was no longer directly detectable (4). Figure 4 illustrates the general protocol in which tissue spleen cells were used following cultivation for 5 days in vitro either restimulated or not (immune). Thirty million of these cells were then transferred to recipient nude mice which had been infected with 10 lethal doses$_{50}$ of either A/Port Chalmers/1/73 or A/PR/8/34 virus one day earlier.

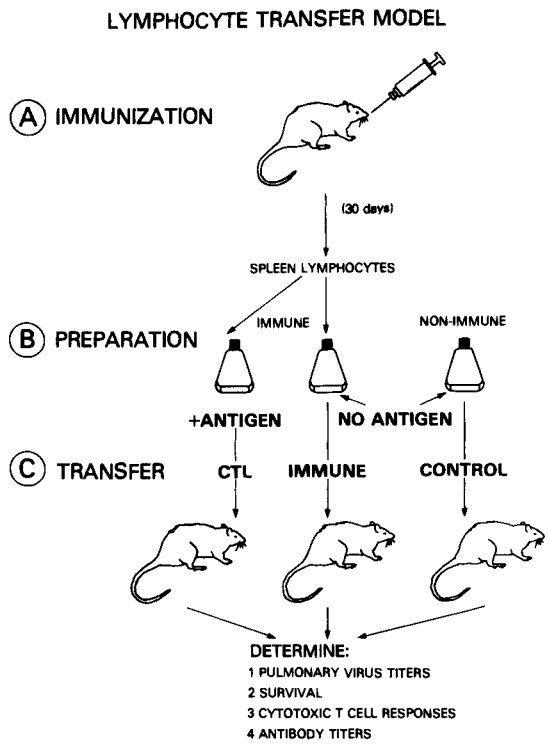

Figure 4. A graphic representation of the methods used to restimulate spleen cells in vitro prior to transfer to infected mice.

The protection conferred by these cells is shown in Table 1. There were no survivors in the groups which were infected and did not receive spleen cells or in the groups which were infected and received spleen cells stimulated by B/Hong Kong virus. Mice which received stimulated immune spleen cells after challenge with homologous virus were best protected, and those that received stimulated immune spleen cells after heterologous type A challenge were also significantly protected, but to a somewhat lesser degree. Thus, it is clear that cross-reactive protection was afforded by immune spleen cells which were restimulated *in vitro* prior to transfer, and that the highest degree of protection was afforded by transfer of immune spleen cells which had been restimulated with homologous virus prior to transfer.

TABLE 1. Survival of Nude Mice after Transfer of Secondarily Stimulated Lymphocytes

Challenge Virus	Transferred Lymphocytes[a]			
	A/Port Chalmers	A/PR/8	B/Hong Kong	None
A/Port/ Chalmers/ 1/73 (H3N2)	60 ($p<0.01$)[b]	40	0	0
A/PR/8/34 (HON1)	67 ($p<0.001$)	87 ($p<0.001$)	0	0

[a]The percentage of nude mice surviving on day 30 post infection is given. This is calculated from groups of 15 mice infected with 5 lethal dose$_{50}$ and receiving 30 x 10^6 spleen lymphocytes from BALB/c mice which were restimulated *in vitro* for 5 days with the homologous virus and then transferred to nude mice 1 day after infection, or from infected mice receiving no lymphocytes.

[b]Significance was determined by chi-square comparison to mice infected with the same virus and receiving no lymphocytes.

The cross-reactive protection observed was consistent with cytotoxic activity of immune spleen cells following secondary stimulation *in vitro*. Spleen cells from mice immunized *in vivo* with virus and then restimulated *in*

in vitro 30 days later kill target cells across subtype barriers to a greater degree than cytotoxic spleen cells which were induced during primary infection (Ennis and Wells unpublished data, 6,7). Thus the specificity of the cytotoxicity observed *in vitro* was reflected in the protection these cells conferred to challenge *in vivo*. These cytotoxic spleen cells have been demonstrated to be H-2 restricted in their killing, and the killing is due to T cells, so they are cytotoxic T lymphocytes.

DISCUSSION

The experiments described above indicate that spleen cells of donor mice infected with influenza one month earlier can be restimulated *in vitro* and can effect recovery of nude mice with influenza pneumonia. The results confirm earlier experiments performed in our laboratory (2,3). In addition these results also confirm that donor immune spleen cells which are not restimulated with viral antigen prior to transfer do not aid in recovery, despite their helping the recipient nude mice develop very high levels of antibody, similar to the results noted by us earlier (3). Recovery of nude mice from influenza pneumonia is therefore accomplished by immune spleen cells which have high cytotoxic T cell activity, but not by immune spleen cells which enhance antibody development.

In the present investigation we also analyzed the antigenic specificity of the protection mediated by these immune spleen cells. At the time of their removal 30 days after infection, they do not possess detectable cytotoxic T cell activity, but following stimulation with viral antigen *in vitro* for 5 days they become highly cytotoxic. The specificity of this secondarily *in vitro* stimulated cytotoxic T cell for target cells *in vitro* is specific for Type A viruses but cross-reactive among the A subtypes, as reported by Effros *et al.* (6) and Zweerink *et al.* (7). In addition, when these *in vitro* stimulated immune spleen cells are transferred to recipient nude mice with influenza pneumonia, they effect recovery in mice infected with the same or another A subtype virus, but not against Type B virus. The protection appears to be greatest against the homologous virus but is also highly significant against a heterologous A strain. These data confirm and extend our earlier reports which indicate that immune spleen cells with enhanced cytotoxic T cell activity are associated with recovery of mice from influenza pneumonia, and the specificity of this effect is similar to the specificity of the cytotoxic T cells

detected *in vitro* at the time of cell transfer.

ACKNOWLEDGMENTS

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REFERENCES

1. Ennis, F.A., Wells, M.A., Butchko, G.M. and Albrecht, P. (1978). J. Exp. Med. 148, 1241.
2. Wells, M.A., Albrecht, P. and Ennis, F.A. (1981). J. Immun. 126, 1036.
3. Wells, M.A. and Ennis, F.A. (1981). J. Immun. 126, 1042.
4. Ennis, F.A., Martin, W.J., Verbonitz, M.W. and Butchko, G.M. (1977). Proc. Nat. Acad. Sci. 74, 3006.
5. Ennis, F.A., Martin, W.J. and Verbonitz, M.W. (1977). J. Exp. Med. 146, 893.
6. Effros, R.B., Doherty, P.C., Gerhard, W. and Bennink, J. (1977). J. Exp. Med. 145, 557.
7. Zweerink, H.J., Courtneidge, S.A., Skehel, J.J., Crumpton, M.J. and Askonas, B.A. (1977). Nature 267, 354.

AN INFLUENZA IMMUNOSOME: ITS STRUCTURE AND ANTIGENIC PROPERTIES. A MODEL FOR A NEW TYPE OF VACCINE

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1. ABSTRACT

The current influenza virus vaccines made of inactivated particles often induce undesirable local and general pyrogenic reactions particularly among youngsters. We have made a selective extraction of membrane glycoproteins and constructed an immunosome which is exclusively made of hemagglutinin and neuraminidase attached onto the membrane of a preformed liposome. The phospholipids were solubilized with the detergent β-D-octylglucoside and the liposomes were formed by the injection method. The hemagglutinins were extracted from purified influenza viruses with Triton N-101. The solubilized glycoproteins were purified by centrifugation through a discontinuous sucrose gradient; the non-dialysable Triton was exchanged during the centrifugation for β-D-octylglucoside that was incorporated into the gradient. When the hemagglutinin subunits, free of detergent, were mixed with preformed liposomes, the incorporation on the surface membrane was extremely low. This difficulty could be overcome by mixing a protein-detergent complex with preformed liposomes, the membranes of which had been "fluidified" in the presence of minute amounts of detergent. A decreasing detergent gradient dialysis allowed the exchange of the proteins from detergent to the phospholipid bilayer. This technique recreates a structure that is almost indistinguishable from the virus particle. This immunosome is highly immunogenic and possesses an hemagglutinating activity much greater than the viral subunits.

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2. INTRODUCTION

Vaccination with inactivated whole influenza viruses is effective but often induces undesirable side effects which limit their administration, particularly to youngsters. These complications are generally caused by toxic components in the virus preparations. Therefore, there is a need for a vaccine that would not contain the substances responsible for these side effects. This can be achieved by eliminating viral lipids, nucleic acids and proteins other than the antibody-inducing surface glycoproteins. The ideal vaccine should contain only the necessary antigenic subunits, which in influenza, are the hemagglutinin and neuraminidase. But, although subunit vaccines have the great advantage of being devoided of side effects, they seem to be much less immunogenic than whole viruses (Wright et al., 1976; Gross et al., 1977). Preparations of influenza hemagglutinin in its monomeric form, obtained by releasing the hydrophilic part using a protease, was shown to be poorly immunogenic in hamsters and man (Brand 1972; Jennings et al., 1974; Tyrrell 1974). On the other hand, our results indicate that a vaccine made of a solution of hemagglutinin rosettes is also much less immunogenic than vaccines made with whole viruses. Perhaps the form in which the proteins are presented in the rosette aggregates could be such that they interact poorly with the cells of the immune system.

In most enveloped viruses, the spike glycoproteins are anchored in the membrane by their hydrophobic rich amino acid sequences. Many reports were presented on the reconstitution of glycoproteins of various viruses in liposomes (Helenius et al., 1977; Morein et al., 1978; Petri and Wagner 1979; Huang et al., 1979; Hsu et al., 1979; Miller et al., 1980). The method applied by these authors essentially consisted in solubilizing the phospholipids and proteins in detergent, mixing the two components together and removing the detergent by dialysis. This procedure, which consists in forming the liposomes in the presence of proteins, necessarily leads to the entrapment of a large amount of proteins inside the liposomes and concomitant loss of immunogenic material.

In this paper, we describe a method of inserting influenza hemagglutinin and neuraminidase molecules on the outer surface of preformed liposomes. This technique recreates a structure almost indistinguishable from the viral particle that is highly immunogenic and that posesses an hemagglutination activity much greater than the viral subunits. We have called this structure immunosome.

3. MATERIALS AND METHODS

A. *Purification of Viruses*

Virus pools were prepared by allantoic inoculation of 10-day-old embryonated eggs. Allantoic fluid, harvested three days later, was concentrated by molecular filtration with the Pellicon Cassette System (Millipore PSVP 000 01 membrane) and purified on a 15% to 60% (w/w) linear sucrose gradient. Virus strains A/Bangkok/1/79 (H3N2) and A/England/864/75 (H3N2) X-49 were obtained from the Bureau of Biologics, Department of Health, Education and Welfare, Public Health Service, Food and Drug Administration, Bethesda, Maryland. U.S.A. All manipulations were carried out at 4°C unless otherwise stated.

B. *Preparation of HA and NA Antigens*

Purified virus suspension (2 to 4 mg proteins/ml) were solubilized at 22-25°C in phosphate-buffered saline solution (PBS) pH 7.4, containing 0,05 M NaCl and 1% Triton N-101. The solubilized virus was layered onto a sucrose gradient consisting of three zones: on top a 1 ml 10% (w/w) sucrose zone with 0,5 % Triton N-101, in the middle a 10 ml linear 15% to 25% sucrose gradient containing 30 mM β-D-octylglucoside (Calbiochem-Behring Corp. La Jolla, CA. U.S.A.), and at the bottom a 1 ml 60% sucrose cushion containing 30 mM β-D-octylglucoside. Every solution contained 0,5M NaCl. After 24 hours of centrifugation at 4°C, (100 000 g SW 40 Beckman rotor), fractions (1 ml) were collected from the bottom and dialysed against PBS. The amount of protein in each fraction was measured according to the method of Lowry et al. (1951). Presence of Triton was revealed by measuring its absorbance at 276 nm. Hemagglutination and neuraminidase titres were assayed according to Palmer et al. (1975).

C. *Formation of Immunosomes*

Phospholipids were obtained from Avanti Biochemicals Inc., Birmingham, Alabama. Phosphatidylcholine, cholesterol and lysolecitin, at a molar ratio of 8:1:0,5, were solubilized in 50 mM β -D-octylglucoside; 10 mM Tris-HCl pH 7,4. The liposomes were formed by a slow injection of the phospholipid solution in PBS, dialysed in the same buffer and purified on a small Sephadex G-100 column made in a 10 ml syringe and centrifuged at 600 g for 15 seconds.

The protein solution (HA and NA) was diluted to 700 µg/ml and β-D-octylglucoside was added to a final concentration of 7.5 mM. The mixture was allowed to stand at 22°C for 10 minutes.

The liposome suspension was made 1 mM in β-D-octylglucoside and incubated 10 minutes at 22°C. The "fluidified" liposomes were then mixed with the protein-detergent complex and the immunosomes were formed by a dialysis in a linear decreasing detergent gradient made in a classical two chamber 500 ml gradient former. The gradient was 7.5-0 mM, with respect to β-D-octylglucoside, in PBS and was performed in 48 hours. When the gradient became zero, the suspension of immunosomes was dialysed against PBS.

D. *Immunological Studies*

Groups of ten mice (Crl: CD ^{R} -1 (ICR) BR) 20-22 g were injected intraperitoneally with 0.5 ml of a suspension of immunosomes, intact viruses or subunits. The quantity of HA (ug/ml) was estimated by single-radial-immunodiffusion (William et al. 1980). Serum samples were taken after 14 days. Antibodies to HA were quantified using the hemagglutination inhibition test according to Palmer et al. (1975).

4. RESULTS

A. *Purification of Glycoproteins*

The purified viruses were treated with Triton N-101 in order to remove the hemagglutinin and the neuraminidase from their membrane. The solubilized glycoproteins were purified by isopycnic centrifugation on a non-linear sucrose gradient; the non-dialysable Triton was exchanged during the centrifugation for the dialysable β-D-octylglucoside that was incorporated into the gradients. Fig. 1 shows that the glycoprotein-β-D-octylglucoside complex banded at 20% sucrose, whereas the lipid and Triton banded at 10%, the core sedimented down to the 50% sucrose cushion.

The HA and NA fractions were pooled and dialysed against PBS. Fig. 2 shows the purified hemagglutinin and neuraminidase as rosettes, which is the structure they exhibit in aqueous solution, indicating that the Triton was efficiently exchanged for β-D-octylglucoside during the centrifugation. The complete removal of Triton is essential in order for biological activities to be tested.

Our results indicate that as little as 0.01% Triton causes hemolysis of red blood cells in the HA test. Because of its particularly high critical micellar concentration (25 mM) that renders it readily dialysable, β-D-octylglucoside is well suited for the solubilization of membrane proteins. Nevertheless, we have used Triton instead of β-D-octylglucoside for the solubilization of the viruses because the former gives better yields.

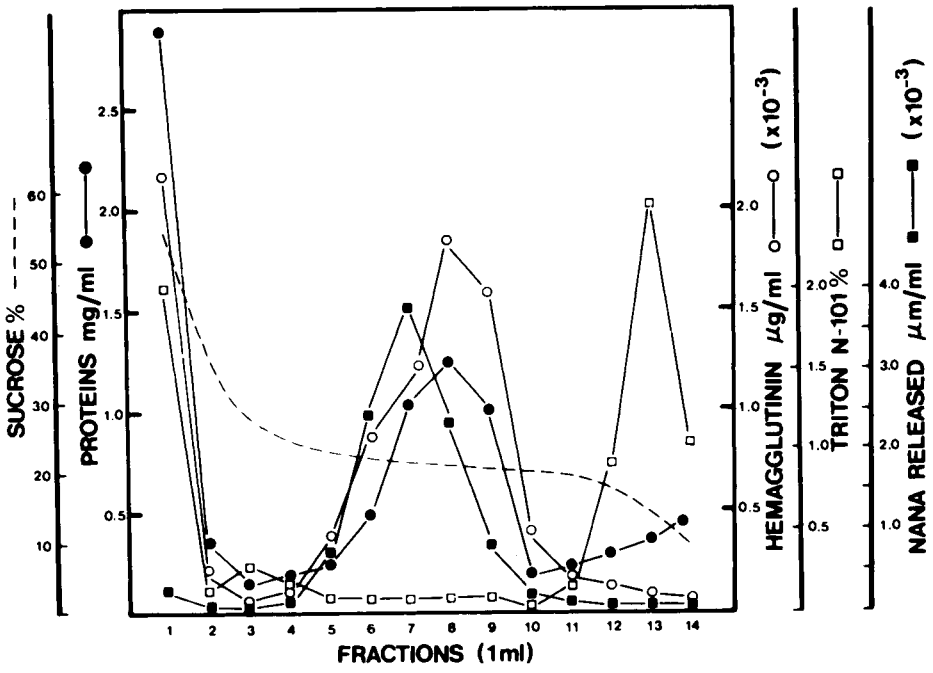

FIGURE 1. Purification of HA and NA on a non linear sucrose gradient. Gradient were fractioned from the bottom. Each fraction was dialysed against PBS and its protein concentration was measured according to Lowry et al., (1951). HA and NA titers were assayed according to Palmer (1951). The % of Triton was estimated by absorbance at 276 mm using a protein solution as a blank. Proteins ●—● HA o—o , NA ■—■ , Triton % □—□ , Sucrose % – – – .

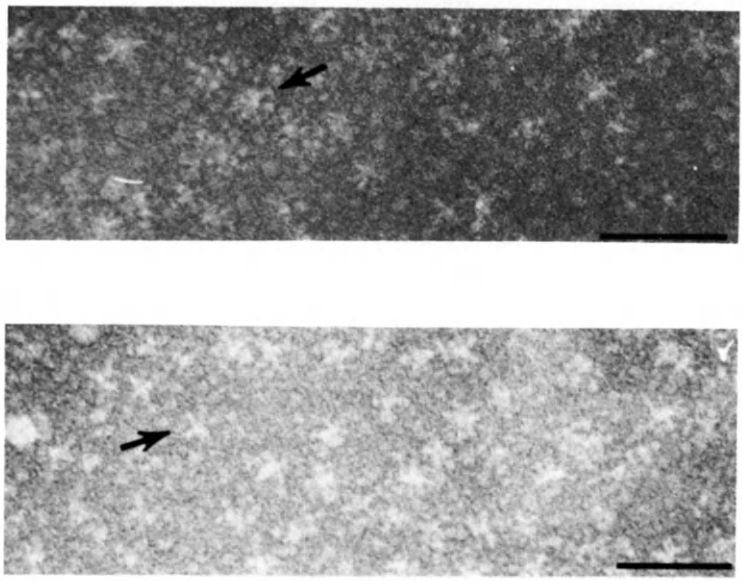

FIGURE 2. Electron micrographs of HA and NA rosettes negatively stained with 3% phosphotungstic acid. The arrows point at neuraminidase rosettes; the bar represents 100 nm.

B. *Anchorage of Viral Antigens on the Outer Surface of the Liposomes Membrane*

To make an immunosome three steps are required: a) the formation of liposomes; b) the formation of a protein-detergent complex; c) the insertion of the glycoproteins in the phospholipid bilayer. The liposomes were formed by the injection method and further purified on a small Sephadex column. We have compared this technique with the one that consists in solubilizing the phospholipids in organic solvents, evaporating the solvent and submitting the multilamellar film suspension to sonication as described by Almeida (1975) and Oxford (1981). We found that the liposomes obtained by the injection method were mostly unilamellar and relatively homogeneous in size, ranging from 50 to 100 nm in diameter (Fig. 3). The liposomes formed by sonication were multilamellar and very heterogeneous in size.

When hemagglutinin and neuraminidase rosettes were mixed with preformed liposomes, the incorporation of the glycoproteins in the membrane was extremely low, likely because the hydrophobic tails of the HA and NA molecules were not available for anchorage, being engaged in the rosette formation. This difficulty could be overcome by forming first a protein-detergent complex that was mixed with preformed liposomes, the membrane of which had been

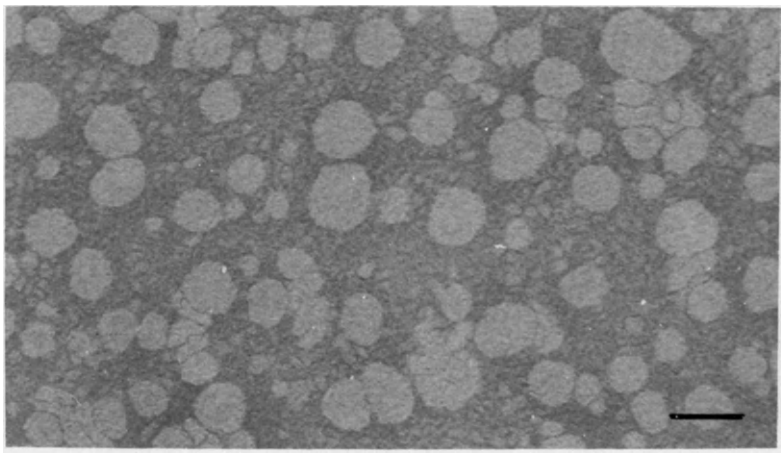

FIGURE 3. Electron micrograph of negatively stained unilamellar liposomes prepared by the injection method (for details see Materials and Methods); the bar represents 100 nm.

"fluidified" with minute amounts of detergent. The insertion of the glycoproteins in the liposome membrane was achieved by exchanging the detergent bound to the protein for the phospholipid bilayer by a slow elimination of the detergent during the decreasing detergent gradient dialysis. Ideally, the slope of the gradient should be such that the rate of liberation of HA and NA molecules would not permit the formation of rosettes; in order to satisfy their hydrophobicity, the free HA and NA molecules will anchor in the phospholipid bilayer. Fig. 4a shows that this technique recreates a structure very similar to the viral particle (Fig. 4b) where the hemagglutinin and neuraminidase spikes are inserted in the membrane forming a fringe around the particle. A microphotograph taken at a greater magnification (Fig. 5) shows that the hemagglutinins are really anchored in the phospholipid bilayer such as to give the proteins the same orientation as in the viral membrane.

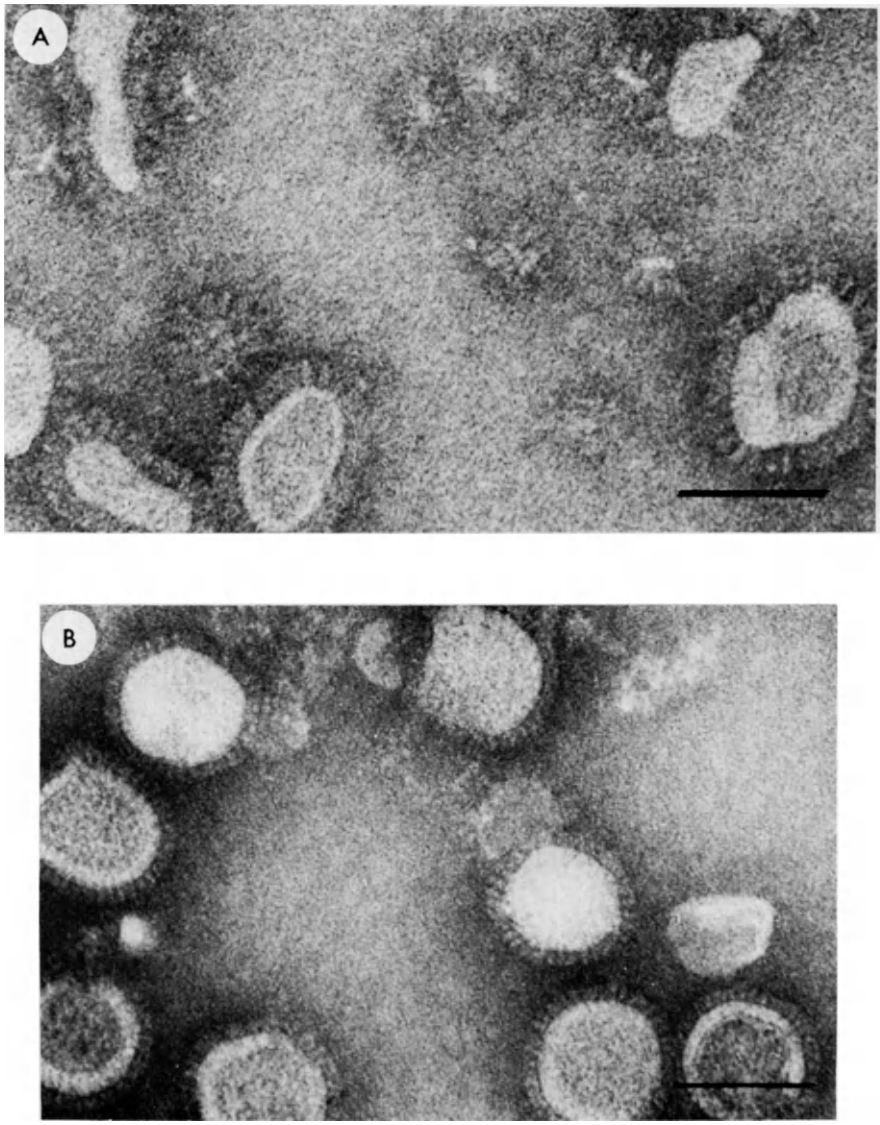

FIGURE 4. Electron micrographs of
(a) immunosomes and
(b) purified viruses (strain A/England/864-X49) negatively stained with 3% phosphotungstic acid. The bar represent 100 nm.

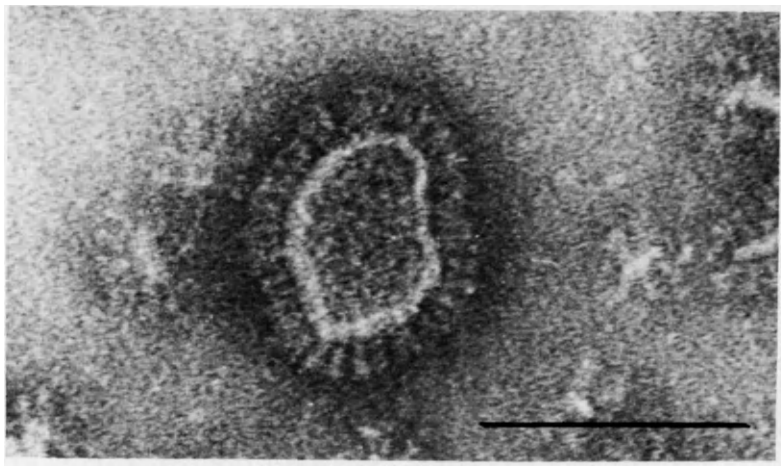

FIGURE 5. Electron micrograph of immunosomes negatively stained with 3% phosphotungstic acid. The glycoproteins were extracted and purified from the virus strain A/England/864-X49. The bar represents 100 nm.

In order to check this, we have looked if the immunosomes possess some of the biological activities of the virus, namely hemagglutination activity and immunogenicity.

C. *Biological Activities of the Immunosomes*

In a first trial, we have compared the hemagglutination activity of the immunosomes with that of the purified proteins used to make them (Table I). The specific activity of the immunosomes was more than eighty times higher than that of the subunits, indicating that the conformation of the proteins in the immunosome is such that the HA sites are well preserved.

TABLE 1. Comparison of the hemagglutination activity of the immunosomes with the viral subunits.

	ug Protein /50 µl	*HA Titer* $\log_2$*	*Specific Activity HAU**/mg Proteins*
Subunits	*19*	*12*	*215,000*
Immunosomes	*6*	*20*	*175,700,000*

* *Average of 15 determinations*
** *HAU: hemagglutinin units*

To test the immunogenicity, groups of mice have been inoculated intraperitoneally with nine different doses of intact virus, HA subunits or immunosome preparations. After two weeks, the animals were sacrificed and their sera were assayed for the presence of antibodies inhibiting hemagglutination. Higher responses were detected in animals immunized with intact virus preparations. However, the increased immunogenicity of the immunosomes compared to the HA subunits indicates that the form in which the proteins are presented in the immunosomes is adequate for eliciting a good immune response. With all preparations a peak was reached with a dose of 6 µg HA two weeks after immunization (Fig. 6). Yet, it is possible that the immunological response to the immunosomes could have been underestimated since a small amount of free rosettes was always detected in the preparations.

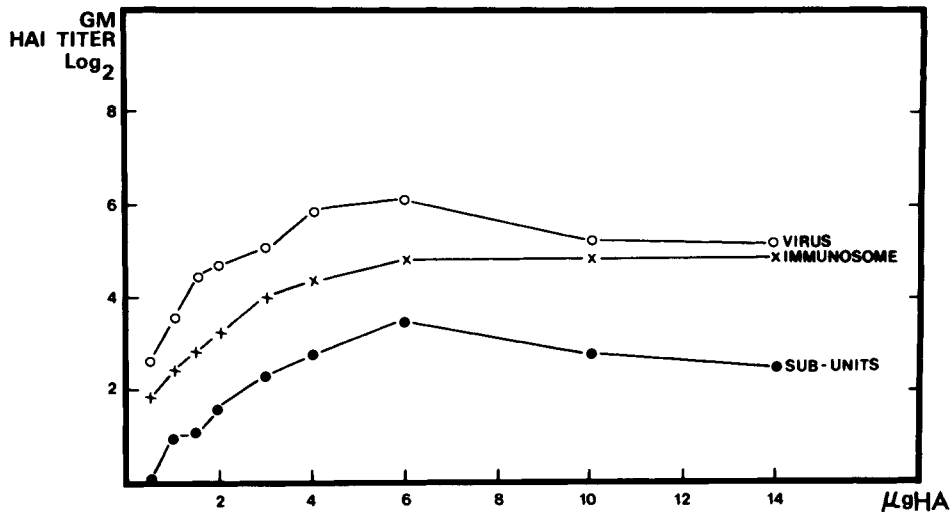

FIGURE 6. Groups of mice were injected intraperitoneally with live viruses, immunosomes or viral subunits at doses ranging from 1 to 14 µg HA. Two weeks later the sera were tested for the presence of antibodies inhibiting hemagglutination.

The immunosomes being intended to be used as vaccines, their stability at 4°C was investigated. Aliquots of 0.5 ml were stored in sterile vials and their hemagglutination activity was monitored over a period of sixty days. No loss of activity was detected during that period. We have no explanation for the increase in hemagglutination activity during the early period of storage. However, we don't think it is accidental since the results presented in Figure 7 are the average of three determinations. Electron micrographs taken at the end of the sixty-day period showed no morphological differences between these immunosomes and the ones in the fresh preparation (Fig. 8).

Other strains of influenza A viruses were used to make immunosomes and the technique was shown to be perfectly reproducible. It is to be noted that the immunological and morphological qualities of the immunosome preparations depend on the degree of purity of the subunits used to prepare them.

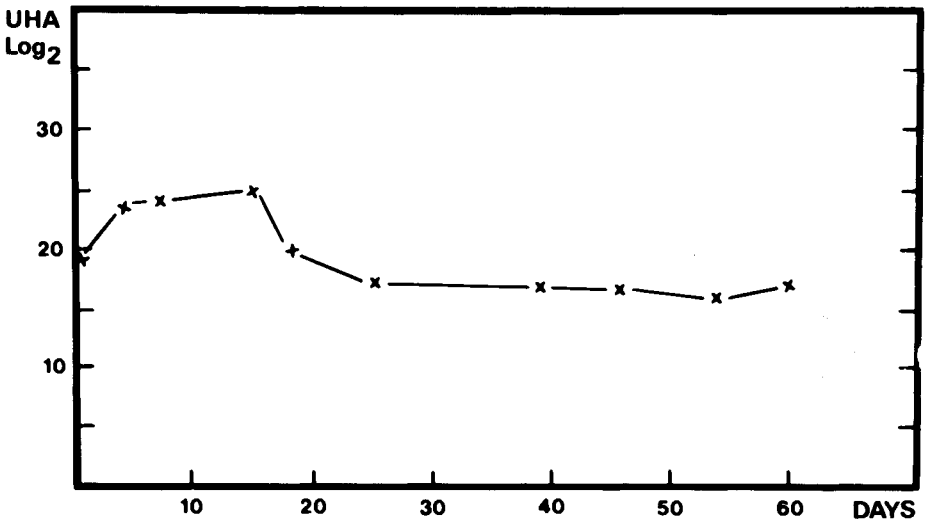

FIGURE 7. Stability of the hemagglutination activity of immunosomes stored at 4°C.

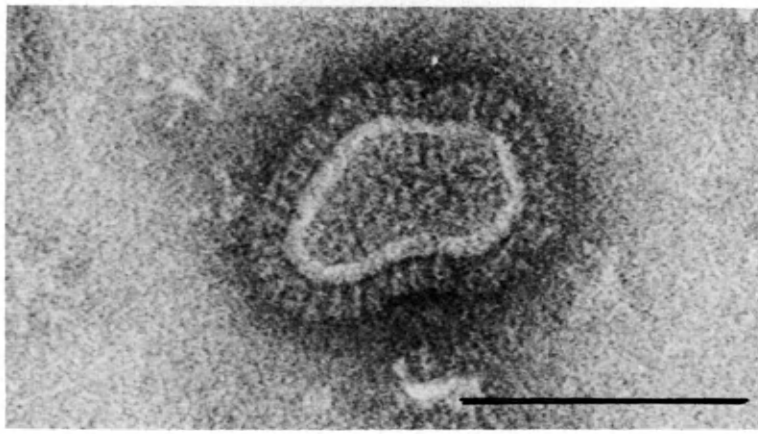

FIGURE 8. Electron micrograph of negatively stained immunosomes (strain A/Bangkok/1/79) kept at 4°C for sixty days. The bar represents 100 nm.

5. DISCUSSION

It is well known that only hemagglutinin and neuraminidase are important for the induction of protection against infection with influenza virus. It is also well established that the physical form of these antigens in a vaccine is of great importance for eliciting an adequate immune response. Monomers of spike proteins were clearly shown not suitable as subunit vaccine. Multimeric aggregates in form of rosettes are more immunogenic than monomers but still much less than whole virus. However, Webster (1976) reported that an influenza subunit vaccine was as immunogenic in man as intact inactivated virus vaccine; it is to be noted that this subunit vaccine was heavily contaminated with nucleocapsids.

Previous studies of the use of liposomes to reconstitute viral glycoproteins have lead to the entrapment of large amounts of the antigens within the internal aqueous compartment of the lipid vesicle. Since our goal is to obtain the most immunogenic structure possible, we have tried to avoid such entrapment by using preformed unilamellar liposomes and attached hemagglutinin and neura-

minidase onto the outer surface of the membrane. The insertion of the glycoproteins in the phospholipid bilayer by the decreasing detergent gradient dialysis seems to be a method of choice much milder than sonication known to be very deleterious to proteins.

The immunosome formed by this technique is nearly as immunogenic as the live virus and much more than the viral subunits. The hemagglutination activity, as well as the morphology of the immunosome were shown to be stable at 4°C, indicating that the insertion and the orientation of the subunits on the liposome surface are the same as on the original virus particle.

The structural and immunological similarities between the immunosome and the viral particle may solve some of the problems encountered with influenza virus vaccines, being exclusively made of purified antigens and naturally occuring phospholipids that can be completely metabolized.

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REFERENCES

Almeida, J.D. (1975). *The Lancet. Nov. 8.*
Brand, C.M., and Skehel, J.J. (1972). *Nature New Biol. 338,* 145.
Gross, P.A. et al. (1977). *J. Infect. Dis. 136,* 623.
Helenius, A., Fries, E., and Kartenbeck, J. (1977). *J. Cell. Biol. 75,* 866.
Hsu, M.C., Scheid, A., and Choppin, P.S. (1979). *Virol. 95,* 476.
Huang, R.T.C. et al. (1979). *Virol. 97,* 212.
Jennings, R. et al. (1974). *Med. Microbiol. Immunol. 160,* 295.
Lowry, O.H. et al. (1951). *J. Biol. Chem. 193,* 265.
Miller, D.K. et al. (1980). *J. Cell. Biol. 84,* 421.
Morein, B. et al. (1980). *Nature 276,* 715.
Oxford, J.S. et al. (1981). *J. Gen. Virol. 52,* 329.
Palmer, D.F., Coleman, M.T., Dowdle, W.R., and Schild, G.C.(1975). "Advanced laboratory Techniques for Influenza Diagnosis". Immunology Series No. 6. U.S. Department of Health, Education and Welfare Public Health Service.
Petri, W.A., and Wagner, R.R. (1979). *J. Biol. Chem. 254,* 4313.

Tyrrell, D.A. (1974), *J. Infect. Dis. 129*, 766.
Webster, R.G. (1976). *J. Infect. Dis. 134*, 48.
Williams, M.S. et al. (1980). *J. Biol. Stand. 8*, 289.
Wright, P.F., Dolin, R., and Lamontagne, J.R. (1976). *J. Infect. Dis. 134*, 633.

GENETIC APPROACHES TO THE PREVENTION OF INFLUENZA A VIRUS INFECTION

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Although inactivated influenza virus vaccines as now formulated are effective, they do not provide complete protection, nor do they appear to retain their effectiveness when administered annually (1,2). For this reason, there is renewed interest in the development of a live, attenuated vaccine that would mimic natural infection in its greater, broader and more durable immunity. In this regard, it is likely that the ultimate live vaccine strains will bear specific identifiable attenuating genes because the genetic basis for attenuation would then be known and could be monitored directly during all phases of vaccine development, manufacture, and utilization in man. It is the goal of current research in this area to identify or produce attenuating genes that will confer a satisfactory level of attenuation upon any reassortant virus into which they are transferred by genetic reassortment. Some success has been achieved using three different approaches.

Host Range Restriction

The first approach involves the use of a donor human influenza virus that has been passaged many times in eggs leading to the emergence of a host range (hr) mutant that grows poorly in man and that does not cause symptoms of respiratory disease. Transfer of hr genes by genetic reassortment has been used by investigators in Belgium and the UK in an attempt to attenuate new epidemic influenza A viruses (3-7). The relationship of genotype to virulence of reassortant viruses derived by mating the avirulent influenza A/PR-8/34 (H1N1) virus and a series of virulent influenza A H3N2 wild-type viruses were genotyped by Florent and his observations provide an interesting insight into one mechanism for achieving attenuation (Figure 1) (8). Reassortant England/69 clone 6 retained moderate virulence although it received all 6 non-surface genes from its PR-8

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parent. Since the PR-8 virus is avirulent for man, it is likely that the hemagglutinin and/or neuraminidase genes of the PR-8 parent also bear significant hr mutations that are required for complete attenuation (9). In contrast to clone 6 that derived all of its P genes from PR-8, six other independently derived H3N2 reassortants were suitably attenuated for seronegative individuals. Each of the latter reassortants possessed a mixed P gene constellation with one P gene derived from the H3N2 parent and the other two P genes derived from PR-8. It should also be noted with respect to non-surface genes that 3 of these reassortants differed from clone 6 only at the RNA 2 locus. These observations suggest that incompatibility of P genes may

GENOTYPE OF PR8 X H3N2 WILD TYPE INFLUENZA A REASSORTANT VIRUSES—RELATIONSHIP TO VIRULANCE FOR MAN

RNA segment number	Gene product	Parental origin of genes in reassortant virus derived by mating PR8 virus and the indicated H3N2 wild type virus						
		England/69 Clone 6	England/69 Clone 64c	England/69 Clone 64d	England/72 "Alice"Δ	Scotland/74 RIT 4025Δ	Victoria/75 RIT 4050Δ	Alaska/77 RIT 4199Δ
1	P1	□	□	□	□	□	□	□
2	P2	□	■	■	■	■	■	■
3	P3	□	□	□	□	□	□	□
4	HA	■	■	■	■	■	■	■
5	NA	■	■	■	■	■	■	■
6	NP	□	□	□	■	□	■	□
7	M	□	□	□	□	□	□	□
8	NS	□	□	■	□	□	□	□
Virulence for man		+	0	0	0	0	0	0

□ = gene derived from PR8 virus.

■ = gene derived from H3N2 wild type virus.

Δ = reassortant virus also gamma inhibitor resistant

Data from Florent, Archives of Virology, *64*, 171-173, 1980.

Figure 1.

lead to satisfactory attenuation of influenza A virus for man. It is perhaps for this reason that a suitable A/USSR/77 (H1N1) x PR-8 (H1N1) reassortant virus has not been described because one would predict that the P gene of these two H1N1 viruses would be compatible. In contrast, satisfactorily attenuated reassortants were recovered when a naturally occurring virulent A/California/10/78 (H1N1) reassortant virus with H3N2 P genes was mated with PR-8 virus (8).

In the future, attenuation of epidemic or pandemic influenza viruses by transfer of PR-8 genes may require the production of reassortant viruses with a mixed constellation of P genes. This might easily be achieved by using the mixed P gene England/69 clone 6 virus as the donor of attenuating genes and selecting for reassortants that possess all six non-surface England/69 clone 6 genes. Parenthetically, the contribution of the gamma inhibitor resistance to attenuation of PR-8 reassortant viruses has not been assessed.

An alternate approach to attenuation involves the use of an avian influenza A donor virus that is restricted in its replication in primate cells. In this situation restriction would be effected by naturally occurring avian influenza virus genes rather than by mutant genes selected by passage of virus in an unnatural host. The scheme now being used to select and characterize naturally occurring avian influenza virus genes that are restricted in primate cells is shown in Figure 2. This work is being carried out in collaboration with Drs. Hinshaw, Webster, London, and Sly.

STRATEGY FOR THE PRODUCTION OF A REASSORTANT VIRUS WITH RESTRICTED HOST RANGE IN MAMMALS.

Step I		Clone several avian influenza A viruses in primary chick kidney culture.
Step II		Identify avian influenza viruses attenuated in squirrel monkeys, hamsters, and ferrets.
Step III	(a)	Transfer to a human influenza A virus all six internal genes of an avian influenza A virus that exhibits restricted replication in mammals.
		or
	(b)	Transfer one or more of the internal avian genes to a human influenza A virus.
Step IV		Evaluate reassortant viruses for level of replication and virulence in ferrets and squirrel monkeys.
Step V		Identify attenuated reassortant virus and use as a donor of its six internal genes to attenuate current human influenza A viruses.

Figure 2.

Initially, viruses recovered from asymptomatic birds were plaque purified and evaluated for evidence of restriction in hamsters and ferrets. Viruses restricted in the lungs of these animals were then studied in squirrel monkeys. Two of the avian viruses studied were clearly restricted in their replication in the lower respiratory tract of the squirrel monkey (Figure 3). Also, these viruses did not produce significant upper respiratory tract symptoms. Of interest, the 2 viruses that were restricted in squirrel monkeys were also enterotropic in ducks, an observation that confirms the avian heritage of these viruses. The avian gene or genes responsible for restriction in primate cells can now be identified by the study of reassortants produced by mating a restricted avian virus with a virulent human influenza A virus.

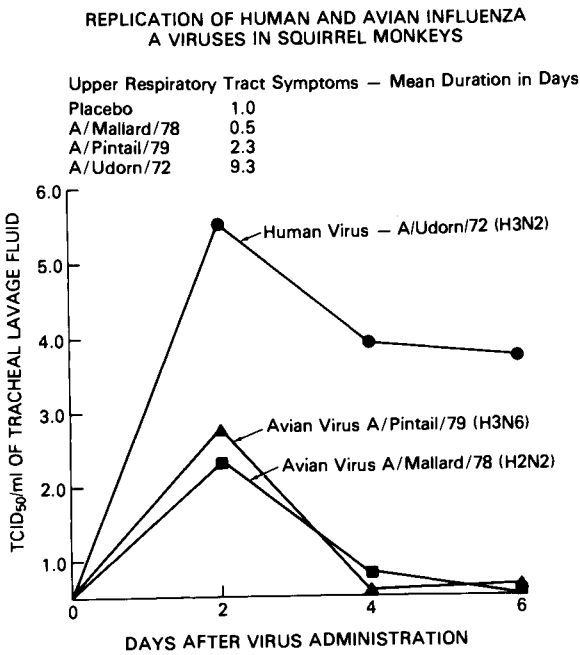

Figure 3. Separate groups of 4 monkeys were administered $10^{6.8}$ $TCID_{50}$ of virus transtracheally. Virus titer was determined on individual specimens and mean $\log_{10}$ titer for the 4 specimens was calculated.

Once the restricting genes and possibly incompatible human-avian gene constellations are identified, an appropriate donor virus can be constructed by genetic reassortment. Many of the influenza genes that have evolved over a long period of time in birds differ significantly from the corresponding genes of human influenza viruses when studied by nucleic acid hybridization (10,11). Because of these marked differences, it is likely that the avian genes will retain their attenuated characteristics after limited replication in man and thereby prove to be stable phenotypically.

Cold-Adapted Mutants

Another technique now being actively pursued in the United States for rapid attenuation of new influenza A viruses involves the use of a donor virus that grows well at a suboptimal temperature that does not support efficient replication of wild-type virus. The A/Ann Arbor/6/60 (H2N2) donor strain was adapted by Dr. Maassab to grow well at 25°C by serial passage at successively lower temperatures (33°C-25°C) in primary chick kidney cell culture (12). This virus was also shown to be *ts* with an *in vitro* shutoff temperature for plaque formation of 38°C (13-15). Genes from this cold-adapted (*ca*) donor strain were transferred to a succession of new antigenic variants of influenza A virus, and reassortant viruses bearing the *ca* and *ts* properties of the attenuated donor virus were isolated. Several reassortants that derived the *ca* and *ts* properties from the A/Ann Arbor/6/60 parent virus were shown to be attenuated for ferrets (16,17). The genes of the A/Ann Arbor/6/60 donor virus responsible for attenuation and for the *ca* and *ts* phenotypes have not been fully identified; however, its P3, P2, and NP genes are present in all A/Ann Arbor/6/60 *ca* reassortants that possess the *ca* and *ts* phenotypes (18). Therefore, it is probable that one or more of these genes represent the site or sites of the *ca* and *ts* mutations.

Ideally, new reassortant viruses produced by mating the A/Ann Arbor/ 6/60 *ca* donor and a new epidemic influenza A virus should receive all the nonglycoprotein genes of the *ca* donor and the surface glycoprotein genes of the new variant. Maassab *et al.* have produced *ca* reassortants with such a gene constellation by mating the A/Ann Arbor/6/60 *ca* donor and wild-type virus at 25°C, a temperature restrictive for replication of wild-type virus (18). Sixty-six percent of *ca* reassortants produced in this manner have the desired gene constellation (18). It is therefore reasonable to

expect that future efforts to produce ca reassortants with all six nonglycoprotein genes from the A/Ann Arbor/6/60 ca parent and the hemagglutinin and neuraminidase genes from the wild-type parents will prove successful.

The six transferable genes present in the A/Ann Arbor/6/60 parent virus were transferred to a succession of reassortant viruses bearing the hemagglutinin and neuraminidase of new antigenic variants (18). The reassortants were then evaluated in susceptible adults in order to determine whether these genes reproducibly conferred a satisfactory level of attenuation on new epidemic wild-type viruses (Figure 4) (16,19-21). The ca reassortant viruses derived from A/Queensland/6/72 (H3N2), A/Alaska/6/77 (H3N2), or A/Hong Kong/123/77 (H1N1) wild-type virus that possessed the desired gene constellation did not induce febrile or systemic illness in any of the 66 seronegative adults who were successfully infected. The few minor illnesses observed were confined to the upper respiratory tract. The corresponding wild-type viruses induced febrile or systemic illness in eight of 14 comparable volunteers. Each of 41 volunteers infected with the A/Hong Kong/123/77 (H1N1) ca reassortant lacked detectable serum antibody to both hemagglutinin and neuraminidase antigens, i.e., these individuals were doubly seronegative at the time of infection.

PARENTAL ORIGIN OF GENES IN COLD-ADAPTED (*ca*) REASSORTANT VIRUSES DERIVED FROM A MATING OF A/A/6/60 *ca* PARENT AND WILD TYPE VIRUS OF THE H3N2 OR H1N1 SUBTYPE – RELATION TO VIRULENCE

RNA segment number	Gene product	Parental origin of genes in *ca* reassortant viruses derived by mating the A/AA/6/60 *ca* virus and the indicated wild type virus				
		A/Queensland/72 H3N2	A/Scotland/74 H3N2	A/Victoria/75 H3N2	A/Alaska/77 H3N2	A/Hong Kong/77 H1N1
1	P3	□	□	□	□	□
2	P1	□	□	■	□	□
3	P2	□	□	□	□	□
4	HA	■	■	■	■	■
5	NA	■	■	■	■	■
6	NP	□	□	□	□	□
7	M	□	□	□	□	□
8	NS	□	■	□	□	□
Shutoff Temperature (°C)		37	39	39	39	37
Virulence for volunteers with serum HI* antibody ≤ 1:8						
a) Serum NI**≤1:2				Low		None
b) Serum NI >1:2		None	Moderate		None	

■ = gene derived from wild type virus.

□ = gene derived from A/AA/6/60 cold-adapted parent.

*Hemagglutination-inhibiting.
**Neuraminidase-inhibiting.

Figure 4.

Thus, any residual virulence specified by the six transferable genes should have been fully expressed. In addition, Dr. Peter Wright and his colleagues observed that the A/Alaska/6/77 (H3N2) and A/Hong Kong/123/77 (H1N1) ca reassortants were satisfactorily attenuated in a limited number of doubly seronegative children (P.F. Wright, personal communication). These results indicate that the six transferable genes present in the A/Ann Arbor/6/60 donor virus can render epidemic influenza A viruses satisfactorily attenuated for fully susceptible individuals.

However, one reassortant, the A/Scotland/74 (H3N2) virus produced moderate illness in susceptible adult volunteers at a dose of $10^{7.5}$ to $10^{8.5}$ $TCID_{50}$. Since this reassortant derived its NS gene from its wild-type parent, it is possible that the NS gene of the A/Ann Arbor/6/60 virus and its reassortants is required for optimal attenuation. To address this question, we studied 3 reassortants produced by mating the virulent A/Alaska/77 (H3N2) virus with the ca donor virus (Figure 5) (20). One reassortant received all 6 transferable genes from the ca donor, while the other 2 reassortants received only 5 of the 6 transferable genes.

PARENTAL ORIGIN OF GENES IN COLD-ADAPTED (*ca*) REASSORTANT VIRUSES DERIVED FROM A MATING OF THE A/ANN ARBOR/6/60 *ca* PARENT VIRUS AND WILD TYPE A/ALASKA/6/77 (H3N2) VIRUS – RELATIONSHIP OF GENOTYPE TO VIRULENCE IN SERONEGATIVE ADULTS

RNA segment number	Gene product	Parental origin of genes in the indicated A/Alaska/6/77 *ca* reassortant		
		CR29 Clone 2	CR31 Clone 3	CR31 Clone 10
1	P3	□	□	□
2	P1	□	□	□
3	P2	□	□	□
4	HA	■	■	■
5	NA	■	■	■
6	NP	□	□	□
7	M	□	□	■
8	NS	□	■	□
Shutoff Temperature (°C)		39	39	38
Virulence ($10^{7.5}$-$10^{7.7}$ $TCID_{50}$)		low	low	low

■ = gene derived from A/Alaska/6/77 wild type virus.

□ = gene derived from A/Ann Arbor/6/60 *ca* parent virus.

Figure 5.

In the latter two instances, the NS or M gene was derived from the wild type A/Alaska/77 (H3N2) virus parent. If the ca donor virus NS or M gene is a major determinant of attenuation, then reassortants that do not receive such a gene should not be optimally attenuated. This was not observed since each of the 3 reassortants was suitably attenuated when evaluated in susceptible adult volunteers. These findings suggest that the A/Ann Arbor/6/60 M and NS genes are not required for satisfactory attenuation of reassortants derived from this ca donor virus. This leaves us with no clear explanation for the observed virulence of the A/Scotland/74 (H3N2) reassortant. Perhaps one or more additional mutations that developed during the reassortment, cloning or passage of this virus were responsible for retention of virulence. Evidence for the occurrence of such mutations was seen during a study of four independently derived A/Alaska/6/77 ca reassortants bearing all six transferable genes of the A/Ann Arbor/ 6/60 ca parent virus (15). These viruses exhibited a greater than one thousandfold difference in plaquing efficiency at 38°C indicating that this phenotypic characteristic of ca reassortant viruses can undergo modification during reassortment and/or passage.

Since the ca reassortant viruses are also ts, it is important to assess the contribution of this property to the attenuation. In previous studies of chemically induced ts mutations, the degree of temperature sensitivity of ts reassortants appeared to be an important determinant of attenuation (22-25). Ts reassortants that had a 37°C or 38°C shutoff temperature for plaque formation were satisfactorily attenuated, whereas ts reassortants with a 39° shutoff temperature produced moderate illness in susceptible adults. In contrast, the A/Alaska/77 ca reassortant CR-29 clone 2 that has a 39° shutoff temperature did not induce febrile illness in adults or children (20). These results suggest that non-ts mutations in the A/Ann Arbor/6/60 donor virus play a major role in attenuation of its reassortants.

During replication in susceptible individuals, most of the ca reassortant viruses were stable genetically (Table 1) (16,19-20). Those reassortant viruses that possessed all 6 transferable A/Ann Arbor /6/60 genes retained the ts and ca phenotypes. However, some children infected with the A/Alaska/6/77 (H3N2) reassortant shed virus with a decreased level of temperature sensitivity. In addition, instability of the ca phenotype was observed with 2 reassortants that received only 5 of the 6 transferable A/Ann Arbor/6/60

GENETIC STABILITY OF COLD-ADAPTED REASSORTANT VIRUSES AFTER REPLICATION IN MAN

Reassortant Genotype				Volunteers	Number of isolates tested	% with indicated phenotype	
Wild type HA and NA genes		Other 6 genes					
		ca	wild type			*ca*	*ts*
H3N2	1972	6	0	Adults	24	100	100
H3N2	1977	6	0	Adults Children*	16 69†	100 not tested	100 100†
H1N1	1977	6	0	Adults* Children*	62 78†	100 not tested	100 100
H3N2	1974	5	NS	Adults	18	61	100
H3N2	1977	5	NS	Adults	3	100	100
H3N2	1977	5	M	Adults	16	100	100
H3N2	1975	5	PI	Adults	6	83	100

*Volunteers were not previously infected with an influenza A virus containing an immunologically related hemagglutinin.

†Nine of the 69 isolates plaqued at 39°C, a temperature for immunizing virus, but were at least 100-fold restricted compared to titer at permissive temperature (34°C)

‡Data from Dr. Peter Wright, Vanderbilt University, Nashville, Tennessee.

Table 1.

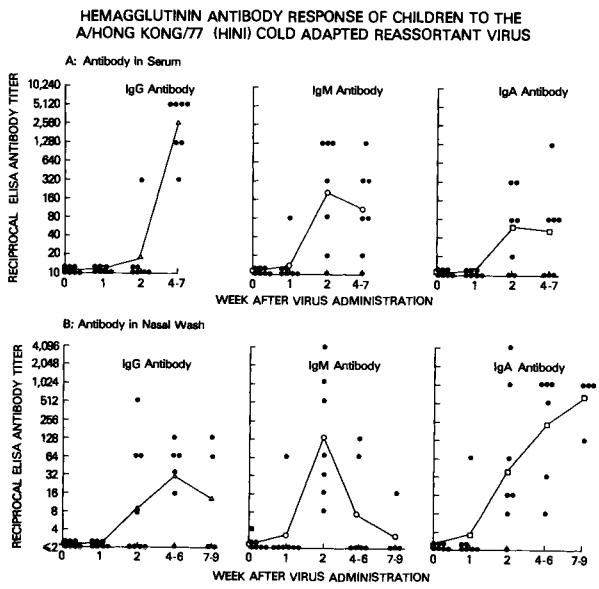

Figure 6.

genes. Thus, some volunteers infected with the A/Scotland/74 (H3N2) or A/Victoria/75 (H3N2) reassortant shed virus that exhibited a decreased effficiency for plaque formation at 25°C. These findings suggest that the potential for genetic instability exists. What is not clear, however, is the effect of such genetic change on attenuation. Perhaps other silent mutations that have been introduced into genes of the ca donor will provide a safety net that prevents reversion to virulence.

Although the ca reassortants are restricted in their replication in the human respiratory tract, these viruses stimulate both a systemic and local immune response. Isotype-specific antibody to the purified hemagglutinin of influenza A virus was measured by ELISA using serum and nasal wash specimens from children infected with a ca reassortant (Figure 6). In collaborative studies with Drs. Nelson and Wright, we observed that doubly seronegative children developed both systemic and local immune responses involving each of the isotypes studied.

Temperature-Sensitive Mutants

In studies performed in our laboratory, chemical mutagenesis was used to produce ts mutations on genes that code for non-surface viral proteins so that the mutant genes could be transferred into reassortant viruses bearing the surface antigens of new epidemic or pandemic strains (26). It is implicit in this approach that chemically induced ts mutations are primarily responsible for the attenuation of reassortant viruses bearing ts genes. This was shown to be the case for reassortants derived from two different ts donor viruses (27,28). The ts genes of the donor viruses were the only genes that were always transferred into ts reassortants that were satisfactorily attenuated for humans (27,28). In one instance, the only genes transferred from the ts-1A2 donor strain to the A/Hong Kong/77 (H1N1) wild-type were its two ts genes. The resulting ts reassortant was satisfactorily attenuated for volunteers who lacked immunity to both viral surface antigens (28). In other studies it was shown that ts genes were responsible for restriction of growth of ts reassortants in the respiratory tract of the hamster (29,30).

The most satisfactory ts donor we developed is the influenza A/Udorn/72 (H3N2) ts-1A2 virus that possesses ts mutations on the genes coding for the P1 and P3 polymerase proteins (29). Transfer of the two ts genes from this donor

into 11 different reassortants yielded viruses with a uniform set of properties: (1) marked temperature sensitivity as indicated by a 37°C shutoff temperature for plaque formation, (2) a 10,000-fold suppression of viral replication in the lungs of the hamster, (3) a 100-fold restriction of viral growth in the hamster's nasal turbinates, (4) genetic stability during viral replication in hamsters, and (5) induction of resistance in hamsters to challenge with wild-type virulent virus (31-33).

Three ts-1A2 reassortants with H3N2 or H1N1 surface antigens were evaluated in adult volunteers who lacked detectable immunity to the hemagglutinin antigen (28). Most of these volunteers also lacked detectable immunity to the neuraminidase antigen. Each of the ts-1A2 reassortants was satisfactorily attenuated and stable genetically. Two of the reassortants were effective in stimulating serum HAI antibodies, while the third reassortant induced a serum antibody response that was detectable by ELISA.

Subsequently the A/Victoria/75 ts-1A2 (H3N2) reassortant was shown by Dr. Peter Wright to be satisfactorily attenuated, antigenic, and genetically stable in completely susceptible children. In contrast, the A/Alaska/77 ts-1A2 (H3N2) reassortant was unstable genetically when tested in a completely susceptible child (34). This child did not develop symptoms, but virus with decreased temperature sensitivity was shed late in the course of infection. This form of genetic instability, that had not been observed previously in tests involving 300 hamsters and 127 seronegative volunteers, represents the central unsolved problem in the ts mutant approach.

The mechanism for genetic alteration leading to loss of the ts phenotype of the ts-1A2 reassortant was studied in some detail during the past year (34). When reovirus ts mutants lose their temperature sensitivity and shift to the ts+ wild phenotype, the usual mechanism involves suppressor mutation rather than true reversion. This was demonstrated by Ramig and Fields, who backcrossed ts+ "revertant" reovirus with wild-type reovirus and recovered ts progeny bearing the original ts mutation (35). We performed a similar analysis with the ts+ influenza virus isolate by mating it with wild-type virus (34). Twenty-two percent of the progeny from this mating were ts, and each ts clone possessed a ts mutation on the P3 gene. This indicated that a new mutation had developed on a gene other than the P3 gene resulting in correction of the temperature sensitivity

of the ts P3 protein. This new mutation was shown to be located on the P2 gene (34). Segregants possessing a ts P1 gene were not recovered from the backcross of the ts+ influenza virus isolated with wild-type virus. Finally, the ts segregants derived from this backcross were less temperature sensitive than ts P3 segregants derived directly from the donor A/Udorn/72 ts-1A2 virus.

These observations indicate that the ts-1A2 reassortant underwent a complex set of genetic alterations during replication in a seronegative child. At least three separate new mutations developed that permitted the highly defective ts-1A2 reassortant to escape its ts phenotype. A new mutation developed on the P3 gene that decreased but did not abolish the temperature sensitivity of its product, the P3 polymerase protein. This could represent intragenic suppression or reversion of one of several ts mutations. A new mutation on the P1 gene, that represented intragenic suppression or reversion, led to loss of temperature sensitivity of its product, the P1 protein. Finally, a suppressor mutation developed on the P2 gene that corrected the temperature sensitivity of the P3 gene product.

The response of seronegative adult volunteers to the ts+ influenza virus was similar to that observed with wild-type virus (M.D. Tolpin, et al., unpublished observation). This suggests that the ts phenotype of the ts-1A2 reassortant is a major determinant of attenuation and that loss of this phenotype leads to restoration of virulence. Genetic instability leading to restoration of the ts+ phenotype and the possibility of spread of ts+ virus to contacts precludes use of ts 1A2 reassortants for immunoprophylaxis, despite the fact that these reassortants are satisfactorily attenuated for highly susceptible individuals.

We have currently produced an additional ts donor virus that appears to be more stable than the ts-1A2 virus in vitro and in vivo and are in the process of evaluating it as a possible donor of its ts genes to attenuate new variants of influenza A virus.

Strategy for Producing More Stable, Defined Mutations in Influenza Virus

It is clear from the foregoing discussion that one of the most urgent needs in experimental immunoprophylaxis is the development of attenuated mutants that are completely stable genetically. The restriction of growth of an

attenuated mutant *in vivo* exerts strong positive selection for new mutations that allow the virus to grow better and escape the limitations imposed by its original mutations. We need stable mutants that are unable to escape from their attenuated phenotype by reversion and/or suppression during replication in completely susceptible individuals.

Theoretically, deletion mutations should be stable because they are not subject to reversion and it is unlikely that they would be easily suppressed by a new mutation at another site on the viral genome. For this reason, we have initiated attempts to produce stable deletion mutations that will render a virus sufficiently defective so that it becomes attenuated but not so defective that it loses viability. This type of genetic surgery can only be performed on DNA. Hence the RNA influenza genes must be transcribed into DNA and then manipulated in this form. This part of the strategy has already been realized by Dr. Lai and his colleagues (36). The intriguing problems that remain to be solved are those of inducing the desired mutations and transcribing the mutant DNA into an RNA form that can be transferred back into an infectious virus.

REFERENCES

1. Meyer, H.M., Jr., Hopps, H.E., Parkman, P.D., and Ennis, F.A. Amer. J. Clin. Path. 70, 146 (1978).
2. Hoskins, T.W., Davis, J.R., Smith, A.J., Miller, C.L., Allchin, A. Lancet 1, 33 (1979).
3. Huygelen, C., Peetermans, J., Vascoboinic, E., Berge, E., and Colinet, G. *in:* International Symposium on Influenza Vaccines for Men and Horses (Symp. Series Immunobiol. Standard), vol. 20, pp. 152 (1973).
4. Lobmann, M., Delem, A., and Jovanovic, D. Develop. Biol. Standard, 39, 43 (1977).
5. McMahon, D., and Schild, G.C. J. gen. Virol. 15, 73 (1971).
6. Morris, C.A., Freestone, D.S., Stealey, V.M., and Oliver, P.R. Lancet ii, 196 (1975).
7. Nicholson, K.G. and Tyrrel, D.A.J. Lancet i, 1309 (1976).
8. Florent, G. Arch. of Virol. 64, 171 (1980).
9. Beare, A.S., Schild, G.C. and Craig, J.W. Lancet ii, 729 (1975).
10. Scholtissek, C. *in:* Current Topics in Microbiology and Immunology, vol. 80, p. 139 (1978).

11. Scholtissek, C. and von Hoyningen-Huene, V. Virol. 102, 13 (1980).
12. Maassab, H.F. Nature, Lond. 213, 612 (1967).
13. Maassab, H.F. J. Immun. 102, 728 (1969).
14. Spring, S.B. Maassab, H.F., Kendal, A.P., Murphy, B.R., and Chanock, R.M. Virol. 55, 233 (1977).
15. Murphy, B.R., Maassab, H.F., Wood, F.T., Jr., and Chanock, R.M. Inf. Immun. (1981) (in press).
16. Davenport, F.M., Hennessy, A.V., Maassab, H.F., Minuse, E., Clark, L.C., Abrams, G.D., Mitchell, J.R. J. Infect. Dis. 136, 17 (1977).
17. Maassab, H.F., Spring, S.B., Kendal, A.P., Monto, A.S. in: "Negative Strand Viruses and the Host Cell" (B.W.J. Mahy and R.D. Barry, eds.). p. 721. Academic Press, New York, (1978).
18. Cox, N.J., Maassab, H.F., Kendal, A.P. Virol. 97, 190, (1979).
19. Murphy, B.R., Holley, H.P., Jr., Berquist, E.J., Levine, M.M., Spring, S.B., Maassab, H.F., Kendal, A.P., and Chanock, R.M. Infect. Immun. 23, 253, (1979).
20. Murphy, B.R., Chanock, R.M., Clements, M.L., Anthony, W.C., Sear, A.J., Cisneros, L.A., Rennels, M.B., Miller, E.H., Black, R.E., Levine, M.M., Betts, R.F., Douglas, R.G., Jr., Maassab, H.F., Cox, N.J., and Kendal, A.P. Inf. Immun. (1981) (in press).
21. Murphy, B.R., Rennels, M.B., Douglas, R.G., Jr., Betts, R.F., Couch, R.B., Cate, T.R., Jr., Chanock, R.M., Kendal, A.P., Maassab, H.F., Suwanagool, S., Sotman, S.B., Cisneros, L.A., Anthony, W.C., Nalin, D.R., Levine, M.M. Inf. Immun. 29, 348 (1980).
22. Murphy, B.R., Chalhub, E.G., Nusinoff, S.R., Chanock, R.M. Infect. Dis. 126, 170 (1972).
23. Murphy, B.R., Chanock, R.M., Levine, M.M., Van Blerk, G.A., Berquist, E.J., Douglas, R.G., Betts, R.F., Couch, R.B., Cate, T.R., Jr. Infect. Immun. 23, 249 (1979).
24. Murphy, B.R., Hodes, D.S., Nusinoff, S.R. J. Infect. Dis. 130, 144 (1974).
25. Richman, D.D., Murphy, B.R., Chanock, R.M., Gwaltney, J.M., Jr., Douglas, R.G., Betts, R.F., Blacklow, N.R., Rose, F.B., Parrino, T.A., Levine, M.M., Caplan, E.S. J. Infect. Dis. 134, 585 (1976).
26. Murphy, B.R., Spring, S.B., Richman, D.C., Chanock, R.M. Postgrad. Med. J. 52, 381. (1976).
27. Markoff, L.J., Thierry, F., Murphy, B.R., Chanock, R.M. Infec. Immun. 26, 280 (1979).
28. Massicott, J.G., Murphy, B.R., van Wyke, K., Huang, K.Y., Chanock, R.M. Virol. 106, 187 (1980).

29. Massicott, J.G., Murphy, B.R., Thierry, F., Markoff, L.J., Huang, K.Y., Chanock, R.M. Virol. 101, 242 (1980)
30. Richman, D.D., Murphy, B.R., Chanock, R.M. Virol. 83, 356 (1977).
31. Murphy, B.R., Hosier, N.T., and Chanock, R.M. Infect. Immun. 28, 792 (1980).
32. Murphy, B.R., Wood, F.T., Jr., Massicot, J.G., and Chanock, R.M. Virol. 88, 244 (1978).
33. Murphy, B.R., Wood, F.T., Jr., Massicot, J.G., and Chanock, R.M. Arch. Virol. vol. 65, 175 (1980).
34. Tolpin, M.D., Massicot, J.G., Mullinix, M.G., Kim, H.W., Parrott, R.H., Chanock, R.M., and Murphy, B.R. Virol. (1981) (in press).
35. Ramig, R.F., Fields, B.N. Virol. 92, 155. (1979).
36. Lai, C.-J., Markoff, L.J., Zimmerman, S., Cohen, B., Berndt, J.A., and Chanock, R.M. Proc. Natl. Acad. Sci. U.S.A. 77, p. 210 (1980).

DEVELOPMENT OF COLD RECOMBINANTS OF INFLUENZA VIRUS AS LIVE VIRUS VACCINES

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ABSTRACT

Cold-adapted (ca) recombinants of influenza viruses were derived, characterized and evaluated in vitro, and in an animal model (ferrets) before use in man as live virus vaccines. The vaccine candidates were found to be attenuated and immunogenic. A set of genetic markers, cold-adaptation (ca) and temperature-sensitivity (ts) were identified which correlated with the level of attenuation. Genetic stability of cold recombinants was proven after administration to ferrets and man. The gene compositions of the vaccine strains were determined, related to the parental types and to the degree of attenuation. Identification of the attenuating lesions has not been fully realized. However, repeatedly the data from in vitro and in vivo studies have shown that cold recombinants (CR) with six genes derived from the cold mutant and the two surface genes from the wild type parent, were predictably attenuated. In these cold recombinants, the ca marker was generally a more reliable indicator of attenuation. Thus, clones having different cut-off temperatures from 37°-39° were equally avirulent in man. In addition, clones which appear to have reverted to (ts^+) in the MDCK cell line are still attenuated and retained the ca and ts markers when evaluated in primary chick kidney cells.

Thus, it can be stated that laboratory criteria are at hand to screen the level of attenuation of candidate live influenza virus vaccine for man. Cold recombinant vaccines

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of influenza virus with six genes from the cold mutants, appear to be the ideal vaccine candidates for use in man.

INTRODUCTION

Live attenuated influenza vaccine has been proposed as a possible alternative to the standard inactivated vaccine because of greater ease of administration and the potential that the immunity induced will be longer in duration. Several methods for attenuation of the virus have been developed. However, the use of attenuated strains for vaccination against influenza virus is hampered by the fact that periodic strain changes will have to be made. In this laboratory great strides have been made in developing live influenza virus vaccine for man with cold-adapted viruses derived by either gradual or abrupt lowering of incubation temperature to 25^{o} or recently by exploiting recombination techniques for rapid update of the vaccine candidates with the relevant surface antigens (1,2,3,4). Thus, it was highly advantageous to develop a genetically stable, molecularly defined, attenuated and immunogenic "Master strain" which can be used in recombination-genetic reassortment with current wild viruses. Clones isolated from this genetic interaction were found to be cold-adapted (ca), had the surface antigen of (HA) of the wild type, while some had the neuraminidase antigen of the cold variant. All showed loss of virulence when tested in animals and man. The cold recombinant vaccines with six genes derived from the cold variant were predictably attenuated when administered to large groups of individuals. The live virus vaccine did not produce any severe reactions, did not transmit to others, and if challenged with the wild strains did result in protection.

RESULTS

Development of "Master Strains" to Types A and B Influenza Viruses

A) Type A Influenza Virus. The initial part of the studies were to adapt influenza to growth at suboptimal temperatures (25^{o}). The method has been followed for selection of attenuated variants, since for many viruses a relationship has been observed between virulence and the ability to grow at lower temperatures (5). The procedure to obtain

cold variants was a step-wise adaptation. The strain A/AA/6/60-H2N2 was chosen because it was isolated in primary chick kidney cells and was found to produce clinical symptoms in ferrets. The line was adapted to growth at 25° by gradually lowering the temperature of incubation 3 degrees at a time until 25° was reached. The strain was propagated in acceptable substrates, embryonated eggs and primary chick kidney cells (PCKC), and was plaque to plaque purified seven times at 25°C. The basis for the plaque purification is to satisfy the following criteria: 1) high yield in tissue culture and embryonated eggs at 25° and 33°C, 2) equivalent efficiency of plaquing at both temperatures, 3) presence of temperature-sensitive marker, since the wild type strain did not form plaques at 25° and did grow at 39°; thus two distinct markers can be used, the ca(cold-adapted) and ts(temperature sensitivity) and 4) behavior in ferrets, an animal model which has been used for approximately 30 years at the University of Michigan for evaluation of pathogenicity and immunogenicity of influenza virus field strains. In collaboration with Dr. Kendal and his colleagues, biochemical analysis followed to help explain the mechanism of the molecular basis for the attenuation of this cold variant, which will allow through genotyping the monitoring of the candidate line in field trials. It is on the basis of the above criteria that the cold variant A/AA/6/60-H2N2 was designated as the "Master strain" and the donor of cold and/or attenuated genes to new wild antigenic variants of influenza viruses.

B) Type B Influenza Virus. A similar procedure was followed for the development of a "Master Strain" cold variant of type B influenza virus. The line designated as B/AA/1/66 isolated in primary chick kidney was adapted gradually to growth and plaquing at 25°C. However, for type B, fewer passages were required at the intermediate temperatures than type A virus and the wild strain was restricted in its growth at temperatures above 36°C. Hence, temperature sensitivity cannot be used as a reliable marker of attenuation for type B influenza virus. Again, this "Master strain" has been used as a genetically stable line and as a donor of cold and/or attenuated genes. A summary of the characteristics of the 2 cold variants is shown in Table I. Thus, it can be stated that these 2 lines have the necessary attributes to provide acceptable live virus vaccine candidates for use in man.

Table I. Summary of the Characteristics of the Donor Cold Variant of Type A and Type B Influenza Virus

	PP*	Titer PFU/ml 25^{o}	33^{o}	Markers	Behavior in man^{o} and ferret**
A/AA/6/60	7X	$5x10_{8}^{7}$	$2x10_{8}^{8}$	ca, ts	immunogenic
B/AA/1/66	5X	$2x10^{8}$	$1x10^{8}$	ca, ts	non-reactogenic

*PP =Plaque-purified
o =References 6,7
** =No evidence found so far of reversion and transmission

Development of Type A Cold Recombinants (CR)

Use of the recombination-reassortment technique has permitted shortening of time required for obtaining seed virus suitable for production of live virus vaccine to six weeks.

To produce a cold recombinant (CR) line, primary chick kidney cells (PCKC) are co-infected with the "Master strain" A/AA/6/60-H2N2 and a contemporary epidemic line of proven virulence using equal multiplicities (5PFU/cell). The method which is referred to as the tube method is designed to derive individual clones with defined genetic characteristics.

In the process of manipulation, selective pressure is applied to the production of independent appropriately attenuated clone(s) by the temperature of incubation at 25°C, at which 1) the wild virus cannot replicate efficiently and 2) by the presence of antibody against the surface antigens of the donor attenuated strain. Emergence of recombinants bearing growth and other characteristics of the A/AA/6/60-H2N2, but with the current hemagglutinin and neuraminidase of the virulent wild type parent is favored. The procedure has been used to derive clones of recombinant lines for biological, biochemical and genetic studies.

Biological Characteristics of Recombinants Derived at 25°C.

I. *In Vitro* Studies. The recombinants derived at 25^{o} were evaluated *in vitro* using two hosts, primary chick kidney cells and embryonated eggs for the following characteristics:

1) The presence of the desired surface antigens. All clones derived at 25^{o} have been found invariably to have the hemagglutinin of the wild parent, while around 10-15 percent of the clones have neuraminidase of the cold mutant.

2) The presence of ca and ts markers. It can be stated that the recombination at 25° have always provided clones possessing both markers. However, in these cold recombinant clones, the shut-off temperature was variable and had alternated from 37°-39°. Development and characterization of 2 distinct cold recombinants which will serve as an example of the diversity of the progeny derived at 25° using the "Master strain" as donor of attenuated genes and distinct wild parental viruses are presented below.

Analysis of clones of the cold recombinant AA-CR22-H3N2/75 are presented in Table II.

Table II Characteristics of Clones of AA-CR22

Virus	Antigenic subtype HA	NA	Shut-off temp.+
A/Vict./3/75-Wild (Lot E-81)	A/Vic.	A/Vic.	39°
x			
A/AA/6/60 Cold mutant	AA/60	AA/60	37°
Cold recombinant clones			
1	A/Vic.	A/Vic.	39°
2	Mixed*		38°
3	A/Vic.	A/Vic.	39°
5	A/Vic.	A/Vic.	38°
6	A/Vic.	A/Vic.	39°
7	Mixed*		39°
9	Mixed*		39°
11	A/Vic.	AA/60	37°
12	A/Vic.	A/Vic.	38°
13	Mixed*		38°
14	Mixed*		39°
15	Mixed*		39°
4,8,14	AA/60	AA/60	37°
16-19	A/Vic.	AA/60	38°

* Mixed = The clones appear to react equally in the HI or NI tests with antisera to both parents
+ Defined as a 100 fold or greater reduction in plaquing efficiency on primary chick kidney cells (PCKC).
HA =Hemagglutinin; NA =Neuraminidase

It is apparent that clones of divergent antigenic properties and cut-off temperature were isolated. Progeny of some

clones appeared to react antigenically in the HI or NI with both parents. Could this be a mixture of two parental types? This seems unlikely since this antigenic heterogeneity persisted for two additional passages and initially the progeny was derived from a single clone. It is possible that this can be interpreted on the basis of true heterozygosis, i.e., of single particles which can give rise to progeny reacting with the two distinct antigens. It was apparent from the results of Table III that this phenotypic character for 2 clones was transient, since upon replating, isolation of clones with antigenic segregation was evident.

Table III. Segregation of Surface Antigens of Two Clones of the Recombinant AA-CR22

Clone 7-HA-Mixed* NA-AA/60	Clone 9-HA-Mixed* NA-AA/60
Passaged and Plaqued at 25^{o}	
Titer: $4x10^{6}$ PFU/ml.	$4x10^{4}$ PFU/ml.
Progeny Selection: 6 clones	9 clones
Antigenic Analysis	Antigenic Analysis
5 clones-HA and NA of AA/60	2 clones-HA and NA of AA/60
1 clone-HA of A/Vic-NA or AA/60	7 clones-HA of A/Vic and NA of AA/60

All derived clones were ca and ts
* Reacted in the hemagglutination-inhibition test with antisera to both parents
HA=Hemagglutinin; NA=Neuraminidase

Thus, heterozygotes are unstable since on replication in the cell they infect, the alleles segregate to yield progeny of 2 parental types. It is intended to determine the gene composition of the rest of the clones of mixed antigenicity, for additional proofs of heterozygosis. The implication of this finding is of importance in terms of genetic heterogeneity of clones derived at 25^{o} and deserve to be evaluated from the practical sense in regard to retention of markers and loss of virulence. Four clones were chosen on basis of antigenic divergence to be evaluated in vivo. The four clones chosen to be evaluated in ferrets had divergent antigenic composition, were cold-adapted and have a shut-off temperature between 38 and 39^{o}C. All clones have HA surface antigen of A/Vict., while only two have 6/60 neuraminidase.

Analysis of the data allowed us to state that regardless of the cut-off temperature and of the antigenic composition,

all 4 clones were equally attenuated and immunogenic in ferrets and retained the ca marker. On this basis, the 2 clones with the HA and NA of the wild type parent were considered as acceptable vaccine candidates for man.

Development and analysis of another cold recombinant, AA-CR24-HswN1, was accomplished using the "Master strain" A/AA/6/60-H2N2 and the wild type A/NJ/8/76-HswN1. Thirty clones were isolated of which sixteen were characterized biologically and the results are shown in Table IV. The clones show differences in antigenic makeup and cut-off temperature. Some of the clones were hybrids possessing the hemaglutinin of A/NJ and the neuraminidase of AA/60, while others were classified antigenically as belonging to subgroup I (Sbg. I) or II which have been identified as subpopulation of the AA/NJ/8/76 wild-type strain (8).

Table IV. Characteristics of Clones of AA-CR24-HswN1.

Virus	Antigenic subtype HA	NA	Shut-off temp.
A/NJ/8/76-"Wild"	A/NJ	A/NJ	39^0
A/AA/6/60-Cold mutant	AA/60	AA/60	37^0
Cold recombinant clones			
1,2,8,15	A/NJ-Sbg I*	A/NJ	39^0
3a,5,7,9,11	A/NJ-Sbg II*	"	39^0
4a	"	"	39^0
12 & 13	"	AA/60 (Hybrid)	38^0
14,16	A/NJ-Sbg I	"	38^0

*Sbg I =Subgroup I, Sbg II =Subgroup II

Thus, the procedure of recombination at 25^0 has furnished clones with diversity in reassortments of genes and with defined markers for biological evaluation in the laboratory and in human volunteers.

II. In Vivo Studies. A variety of animal models such as hamsters, non-human primates, and rats(9,10,11,12) have been used to evaluate the degree of virulence of influenza viruses. However, none has been found to offer a reliable indicator of attenuation which can be equated to human virulence.

In our laboratory, the ferret has been used exclusively as an animal model for study of pathogenesis of influenza virus infection in man (13). The ultimate technical objective is to develop a system that could be employed to

supplant the use of human volunteers for evaluation of attenuation of live virus vaccine. The hypothesis is that ferrets substitute as a host whose reaction to influenza closely simulates the reaction of man. Hence, it ought to be possible to establish correlations between findings in human volunteers and in ferrets which could be used to predict vaccine safety (lack of symptoms, signs, and adherence to a characteristic growth pattern of virus concentration and distribution) and efficacy (frequency and height of antibody response and resistance to challenge with wild type virus). Clones with the desired surface antigens, the high yield at 25^{0} and the defined genetic markers were evaluated. Graded doses of each clone were administered intranasally for evaluation of reactogenicity, reversion (fever, coryza, histopathological examinations of lungs and turbinates) antigenicity and immunogenicity. The ferrets with appropriate matched uninfected controls were observed for 8 days. The results in Table V illustrate a typical experiment, evaluating the response in ferrets to infection using graded dosage ranging between $2x10^{7}$ - $3x10^{2}$ PFU/ml. of the candidate live virus vaccine AA-CR29-H3N2-A/Alaska/77.

The data demonstrate that the ferret infectious dose (FID) was approximately $3x10^{5}$ PFU/ml. The FID of ferrets was determined on the basis of clinical signs, virus shedding, antibody response and protection after wild type challenge. For further evaluation of the ferret's model in screening vaccine candidates, the course of infection was followed using the vaccine candidate AA-CR19-H3N2-A/Victoria/75. Table VI illustrates a comparative study in ferrets infected with a cold recombinant and the two parental strains. It is evident, that in ferrets infected with the wild type parent, high titered virus was shed and for a longer period of time when compared to ferrets infected with either the attenuated donor strain or the cold recombinant vaccine. The corresponding markers were retained in the isolates. The clinical response offered additional proof of the virulence of the wild type parent, while the donor strain and the vaccine line were equally attenuated. Before releasing the vaccine line to the manufacturer (Flow Laboratories) for production and safety testing of a vaccine pool for use in man, the following criteria have to be met: 1) high infectious yield at 25^{o} and $33^{o}C$, 2) failure of the virus to grow in the lungs of ferrets, but evidence of growth in the turbinates, 3) uneventful histopathological findings in the lungs and turbinates of ferrets, 4) no evidence of clinical manifestation, 5) genetic stability of the ca and ts markers after ferret infection and 6) lack of transmission.

TABLE V

Response of Ferrets to Infection with
Clone 2 of AA-CR29-H3N2-Alaska

Dilution Used* to infect ferret	Clinical** signs	Virus Isolation at days p.i.	Antibody response	Response after Challenge Clinical++	Virus shedding
Undil	Slight coryza	1, 2, 3	1024	None	0
10^{-1}	None	1, 2, 3	1024	None	0
10^{-2}	None	1	512	None	0
10^{-3}	None	None	64	None	Day 1
10^{-4}	None	None	16	Slight coryza	Day 1, 2
10^{-5}	None	None	8	coryza	Day 1, 2
Control ferrets	-	-	-	High fever and coryza for 4 days p.i.	

* The titer of the inoculum 3 X 10^{7} PFU/ml
** Clinical signs evaluated were fever, coryza
\+ HI - Hemagglutination-Inhibition test
++ Ferrets were challenged 21 days post infection (p.i.) with 10,000 egg infectious doses

Table VI Response of Ferrets to the Cold Recombinant AA-CR19-H3N2 (A/Vic/3/75-H3N2)

Virus	A/Vic/3/75-E5	A/AA/6/60-7Pi-E3	Cold Recombinant AA-CR19 (A/Vic)
Infectious Dose* (EID_{50}/ml)	$10^{5.5}$	$10^{7.7}$	$10^{7.5}$
Clinical Signs	Fever-3 days Coryza-6 days	None	No fever Coryza-2 days
Virus** Isolations at Days	1,2,3,4 ($10^{4.5}$)	1,2 ($10^{3.0}$)	1,2 ($10^{3.5}$)
Properties of isolates+	WT	ca, ts	ca, ts
Antibody° Response	512	1024	1024

*3 Ferrets in each group given the dose intranasally (IN)
** Isolation from a nasopharyngeal swab taken at daily intervals post-infection (p.i.) In parentheses mixture titer (EID_{50}) at the designated day
+ All isolates, WT=Wild type, ca=Cold adapted ts=Temperature sensitive
o Antibody titer as determined in the HI test

Genetic Characterization

In collaboration with Drs. Kendal and Cox, studies are being pursued for genotypic analysis of attenuated cold recombinants. The selection of a candidate live influenza virus vaccine with defined gene(s) composition will be the essential laboratory guideline to provide the predictability needed to relate loss of virulence to gene constellation (14).

The method of recombination with "Master strain," a genetically stable parent with biological markers and defined gene composition, is ideally suited to the preparation of viruses with reproducible gene composition, thus increasing the probability that recombinants produced in different years will have similar levels of attenuation and minimizng the number of human volunteer studies required. The frequency of gene combination observed in cold-adapted recombination virus is shown in Table VII. The data indicate that the recombination at 25° offers with regularity a high frequency of recombinant lines with defined gene composition which can

be evaluated for loss of virulence in humans. The gene(s) responsible for ca and ts markers have not been fully identified. However, recent studies (14) suggested that the genetic composition of ca recombinants might be restricted, so that the two large genes as well as the NP and M protein were always derived form the cold-adapted (ca) donor line.

Table VII Frequency of gene combinations in cold-adapted recombinant viruses

Parental Viruses		No. (%) recombinants with gene composition**			
Cold Mutant	WT	1	2	3	4
A/AA/6/60(H2N2)	H3N2(24)*	12(50)	4(17)	1(4)	7(30)
A/AA/6/60(H2N2)	H1N1(11)	11(100)	0(0)	0(0)	0(0)
	Total(35)	23(66)	4(11)	1(3)	7(20)

* No. of recombinants

** Gene combinations are:

1= RNA 1, 2, 3, NP M and NS from A/AA/6/60 mutant

2= as 1, except RNA 2 from wild-type parent

3= as 1, except M from wild-type parent

4= as 1, except NS from wild-type parent

The idea is that thecluster of genes responsible for cold adaptation is always transferred simply because of the greater efficiency of replication of the cold mutant at 25°. Recent evidence in our laboratory does not fully justify the above conclusion, since the ca and ts markers were transferred by performing the recombination at nonpermissive temperature of 38° (the shut-off to the cold variant). Clones were derived with equivalent gene composition to the clones derived at 25°C which favored the replication of the donor strain.

The detection of multiple lesions in the A/AA/6/60, as shown recently by Cox and colleagues (15) increases the problems of genetic analysis. The compensation for this, is the possibility that recombinants with genes derived from the "Master strain" are less likely to regain their virulence by reversion when administered as vaccines to man than if they contained genes from a mutant virus having one or two single-step lesions.

Results using an animal model and human volunteers are in agreement with this view, since reversions in the ts and ca markers have seldom been recovered from man and had usually been independent in viruses recovered from ferrets or human volunteers infected with cold-adapted recombinants.

Despite the fact that the genes of the cold-adapted (ca) A/AA/6/60 virus have not been completely defined, it is completely clear that the ca donor strain and

its recombinant viruses are attenuated for animals and man.

Genotyping clones of AA-CR31-H3N2 derived by co-infection with the cold variant and the wild type A/Alaska/6/77-H3N2 is shown in Table VIII.

Table VIII Gene Composition of Recombinant Cold-Adapted Virus, AA-CR31-H3N2 A/Alaska/77

Clone No.	Derivation of genes							
	HA	NA	RNA1	RNA2	RNA3	NP	M	NS
2,13,19,20	0	0	X	X	X	X	X	X
3	0	0	X	X	X	X	0	X
4,16,17 (Hybrids)	0	X	X	X	X	X	X	X
10,12	0	0	X	X	X	X	X	0

0 Indicates genes derived from WT parent
X Indicates genes derived from the "Master strain"

It is evident that the clones with divergent gene composition were obtained. Four clones have the six internal genes from the cold variant and the 2 surface glycoprotein genes from the wild type influenza virus strain; the ideal combination for an acceptable live vaccine line. Three clones have five genes derived from the cold variants, while the remaining 3 clones have only one gene derived from the wild type.

Table IX represents a summary of an in vivo evaluation of the 4 clones of AA-CR31-H3N2 A/Alaska/77 with different gene composition. Biological characterization of 2 clones (2 and 20) with the same gene composition but different cut-off temperatures, demonstrates that in cold recombinants with six genes from the cold mutant, the level of attenuation is not related to temperature sensitivity.

The cold recombinant clones 2 and 20 of AA-CR31-H3N2 with the six internal genes derived from the cold variant A/AA/6/60 have different shut-off temperatures. The shut-off temperature of clone 2 is 39^{o} while that of clone 20 is 38^{o}C. However, both clones were equally attenuated in ferrets and no revertants isolated. Thus, it can be said that in clones of cold recombinants with six genes derived from the "Master strain" (A/AA/6/60) differences in shut-off temperatures were not expressed as differences in reduced virulence.

The cold recombinant clone 10 of AA-CR31-H3N2 with the NS gene derived from the WT parent showed some reactogenicity when used to infect ferrets at a high dose ($10^{8.5}EID_{50}$/ml). However, the virus shed retained the ca and ts markers. The

above observation duplicates the findings in man when a high dose ($10^{8.5}EID_{50}$/ml) of clone 7 of AA-CR18-H3N2 (A/Scot/74) was used(16). Clone 7 of AA-CR18 also has the NS gene derived from its wild type parent. However, in instances where reactogenicity was demonstrated, the infectious dose administered was undiluted infected allantoic fluid with a high infectious titer of the order of $10^{8.5}TCID_{50}$/ml. Thus, the reactogenicity can be attributed to unspecified toxicity, upon administration of a high dose of undiluted infected allantoic fluid, rather than reversion to virulence.

Table IX. In Vivo Characteristics of AA-CR31-H3N2 with Different Gene Composition

	Clones of AA-CR31-H3N2-A/Alaska/77			
	2	3	10	20
Genotype	6 genes(ca)	5 genes(ca) M gene (wt)	5 genes (ca) NS gene (wt)	6 genes(ca)
Phenotype*	39°-ca	39°-ca	38°-ca	38°-ca
Ferret Response	Attenuation	Attenuation (dose dependent)	Attenuation	Attenuation
Viral Amplification**	$10^{8.C}$	$10^{7.0}$	$10^{8.0}$	$10^{8.3}$
Ferret Reponse	Attenuation	Reversion and mild disease	Attenuation	Attenuation

* Phenotype - ts(shut-off temperature)-ca(cold adapted)-wt(wild type)

** Virus recovered from ferrets was amplified by passage in eggs and new sets of ferrets were infected.

The cold recombinant clone 3 of AA-CR31-H3N2 with the M gene derived from the WT parent used to infect ferrets exhibited some reactogenicity and reversion to the ts^+ phenotype. However, in every instance, the ca marker was retained. Another documented observation is that infection of ferrets with ts^+ virus did not induce any clinical manifestations, which might represent suppressor mutation in this clone rather than true reversion. Again, the virus shed was attenuated with retention of the ca marker and reversion to ts^+ phenotype.

For the present if recommendation is to be made, it can be stated that in cold recombinant vaccine lines, the presence of NS and or M genes derived from the wild type would be considered unacceptable and only vaccine lines with six genes from the cold mutant A/AA/6/60 will be considered as suitable live vaccine candidates.

Genetic Stability of the ca Marker. A very important criteria which has been met repeatedly is the genetic stability of the cold recombinant vaccines with six genes derived from the cold mutant. Clones were isolated with ts^+ phenotype when the vaccine line AA-CR29-H3N2 was administered to volunteers or was grown in MDCK cells. However, in both studies, the ca marker was retained. Two clones appearing at 39^o in MDCK cells were picked and a pool was made at 34^o. The two lines were evaluated in vivo and in vitro. The results are presented in Table X. The in vitro studies showed that both lines retained the ca and ts markers in PCKC. Thus, the vaccine line reverted to ts^+ only in MDCK cells but not chick kidney cells (PCKC). In addition, the studies in vivo demonstrate that the reversion to ts^+ in MDCK did not result to reversion to virulence in ferrets.

Table X Genetic Stability Studies of AA-CR29 Clone 2

In Vitro Studies in Pirmary Chick Kidney Cells (PCKC)

Virus Line	Infectivity PFU/ml. at 25^o	33^o	39^o
CR29 - Clone 2* EXE2-MDCK34-MDCK39^o-1	$8x10^6$	$2x10^7$	NP-Lysis at 10^{-3}
CR29 - Clone 2* EXE2-MDCK34^o-MDCK39^o-2	$1x10^7$	$1x10^8$	NP-Lysis at 10^{-3} and 10^{-4}

In Vivo Studies - CR29 - Clone 2 - EXE2-MDCK34^o-MDCK39^o-2

Time p.i.	Infectivity titer (log_{10}-EID_{50}/ml) Turbinates	Lungs
3 days	6.3	< 1.0
8 days	< 1.0	< 1.0

Virus was shed for 7 days p.i.
No fever, nasal discharge or anorexia were evident
Histopathology report was uneventful
Selected isolates from turbinates and nasal swabs were ts and ca
*The two markers ca and ts were retained for both lines

Further confirmation of the above conclusion was made from an isolate which was recovered from a nasal wash on a seronegative child on the 5th day following vaccination with the same live virus vaccine (AA-CR29-H3N2, clone 2). The isolate was obtained from a field trial performed by Dr. Peter Wright and his colleagues at Vanderbilt University. The results indicate that the isolate from the child although was ts^+ in MDCK cell line, retained both ca and ts

markers when titrated in primary chick kidney cells (PCKC). The isolate upon administration intranasally to ferrets did not precipitate any clinical signs and the virus isolated from the turbinates and the lungs of ferrets retained both markers in primary chick kidney cells. Thus, in cold recombinants with six genes constellation from the "Master strain", the ca marker reflects more accurately the level of attenuation of these candidate strains. In addition, studies in host systems, such as MDCK cells with trypsin additive require confirmation in a different host such as PCKC before conclusion can be drawn on reversion and virulence of CR vaccine candidates.

Studies in Volunteer

Candidate recombinant vaccine lines with relevant surface antigens and 1) with defined genes, 2) possessing two markers (ca,ts) and 3) characterized as avirulent in an animal model (ferret), were selected for administration to human volunteers. The studies in volunteers were conducted under NIAID auspices in Maryland, New York, Tennessee and Texas along established guidelines for collection testing of specimens and for evaluation of clinical response.

Twelve different recombinant vaccine lines with differing gene composition and with relevant surface antigens, were developed during the past eight years and were administered to man. In general, all the vaccines given to man were immunogenic with varying degrees of potency. However, when the vaccine cold recombinant line AA-CR18-A/Scotland with the "NS" gene derived from the wild type parent was administered to volunteers, a 30% reactogenicity was evident(16). In this instance, the dose administered was undiluted infected allantoic fluid with an infectious titer of $10^{8.5}$ $TCID_{50}$/ml, both ca and ts markers were retained which suggest that the ca phenotype may not uniformly indicate satisfactory attenuation for man. However, as stated previously, the administration of such a high infectious dose might generate toxicity not directly attributed to the level of attenuation of these CR vaccines.

Recent studies of evaluation of influenza virus cold recombinants in young adults by Dr. Murphy, et al., (17) and Drs. Cate and Couch have shown acceptability of these vaccines. The trials in man using cold recombinant clones to A/Alaska/6/77-H3N2 with differing gene composition is shown in Table XI. The extent of the infectivity of the vaccine to serologic and clinical responses and virus shedding are compared to infection with the wild type parent. It is evident that in all instances the three vaccine lines were attenuated and immunogenic. All the isolates obtained

retained both ca and ts phenotypes. Since the 2 clones with the M and NS genes derived from the wild type were attenuated the study suggests that the M and NS genes are not the major determinants of attenuation. Under closed conditions, recent trials in Vienna by Dr. Reeve, et al., (18) and in Houston by Drs. Cate and Couch using 2 cold recombinants, AA-CR19 and AA-CR22 derived at 25° with the 2 surface antigens from the wild type parent (A/Victoria/75-H3N2) and RNA2 also from the WT have demonstrated again that the CR vaccine strains are suitably attenuated. In the studies at Baylor University, protection against challenge with the wild strain was also demonstrated as shown in Table XII. Challenge with wild type A/Victoria produces protection equivalent to natural infection, while infection in unvaccinated group showed 57% illness.

Table XI Summary of Response of Seronegative Volunteers to A/Alaska/6/77 Cold-adapted Recombinant or Wild Type Viruses

A/Alaska/6/77 virus	Dose($\log_{10}$ $TCID_{50}$)	No. tested	% infected*	% with HAI,NI ELISA test	%with any ill-ness
CR-29 clone 2	7.5	24	75	67	6
CR-31 clone 3	7.7	12	100	83	0
CR-31 clone 10	7.7	17	94	82	6
Wild type	4.2	8	100	88	50

Studies were conducted by Dr. B.R. Murphy and his colleagues
*Evidence of virus shedding and/or antibody response

Studies of Dr. Peter Wright and his colleagues in doubly seronegative children have demonstrated that 2 cold recombinants with six genes derived from the attenuated parent, were satisfactorily attenuated and with minimal side effects. Virus shedding was of low magnitude and there was a cell-mediated immune response(19). Results of separate trials using two different cold recombinant vaccines, AA-CR29-H3N2 (A/Victoria) and AA-CR35-H1N1 (A/HK/23/77) are shown in Table XIII. It is evident that the 2 vaccine lines were infectious, and immunogenic. In addition, no reactogenicity was recorded, transmission to seronegative contact was not observed and the virus shed retained both the ca and ts markers where evaluated in primary chick kidney cells. Thus, in this limited number of young children which are doubly seronegative, the cold recombinant vaccines, with six genes derived from the attenuated donor strain has passed all the

Table XII Summary of Response to Intranasal Inoculation with Wild Type Influenza A/Victoria/3/75-H3N2 Virus by Volunteers Having Prior Exposure

Group Interval	Reciprocal[a] Neutralization Titer range (GMT)	Percent shedding	Percent ≥4-fold Antibody titer increase		Illness Percent** 1-5 days			
			Neut	HI	None	1+	2+	3+
CR19-(N-12)* (19 weeks)	<2-32 (10)	33	8	8	100	-	-	-
CR22-(N-6) (6 weeks)	2-256 (36)	0	17	20	100	-	-	-
Natural infection(N-9) (15-17 months)	8-32 (16)	11	56	0	100	-	-	-
Infection Control (N-28)	2-4 (12)	86	86	63	39	4	0	57

Studies directed by Drs. Cate, Couch and colleagues at Baylor University
* Number of volunteers
** Illness: 1+ = mild URI; 2+ = URI plus systemic symptoms; 3+ = febrile (≥100F) illness
a Interval between vaccination or natural infection and wild type virus challenge. All vaccine virus recipients had had serum neutralizing antibody titers of <1:4 against H3VicNeq1 virus before vaccination, and titers at the time of challenge are given in column 2.

criteria essential for safety and acceptability.

Table XIII Summary of trials of AA-CR29-H3N2 and AA-CR35-HINI in Seronegative Children

Vaccine strain	# of volunteers	Dose $TCID_{50}/ml$	% infected	Ab. rise ≥4 fold	Ill.
AA-CR29-H3N2	10	$10^{6.3}$	100	$10/_{10}$	Minor*
AA-CR35-HINI	11	$10^{6.5}$	74	$8/_{11}$	Minor

Studies conducted under the direction of Peter Wright and colleagues at Vanderbilt University. *Indistinguishable from controls.

The work on the development of a safe live type B vaccine is in its infancy. We have stated earlier that a "Master strain" was developed and recombinant lines were derived. The data available from various laboratories have provided encouraging results. A summary of human volunteer trials is presented in Table XIV.

Table XIV The Candidate Vaccine Lines of Type B Influenza Virus administered to Human Volunteers

Virus line	Derivation	Testing in man	Immunogenicity*
B/AA/1/66	CV-"Master strain"-Stepwise	Common Cold Unit-England	High
B/Tec/1/69	CV-Step-wise	NAMRU-4	Moderate
AA-CR7 (B/HK/8/73xB/AA/66)	Recombi-nant	Different areas	Moderate to High
AA-CR17 (B/AA/1/74xB/AA/66)	Recombi-nant	Overseas	Low
RB-77-SFI (B/Tec/10/77xB/AA/66)	Recombi-nant	Vienna	Moderate

*As evaluated by sero conversion rate and clinical manifestations
High= >75% seroconversion, Moderate= 50-75%, Low= <50%

Human trials were performed in various geographic areas with evidence of lack of reactogenicity and transmissibility to contacts genetic stability and immunogenicity ranging from low with the administration of only one cold recombinant

AA-CR17 to moderate and high with others. The temperature sensitive (ts) marker was an important area which differentiates type B from type A influenza virus. With type B influenza virus, the cut-off temperature cannot be used reliably to measure the level of attenuation. Genetic analysis of the "Master strain" and its recombinant lines is being pursued along the same lines that are being followed to evaluate the molecular basis of attenuation of type A influenza virus.

CONCLUSION

The technique of using cold-adapted(ca) "Master strain" of influenza virus with documented genetic stability and attenuation as donor of attenuated genes has provided reliable *in vitro* characteristics and *in vivo* characteristics (ferrets) to evaluate live virus vaccine for use in man.

The procedure through recombination-reassortment at 25^{o} has offered a rapid and predictable method to update the live vaccine with the surface antigens of influenza viruses when faced with new epidemics.

Gene analysis of these vaccine candidates has allowed us to monitor cold recombinants during all phases of vaccine developemnt from the laboratory to the manufacturer to the trials in man.

The lesions responsible for cold-adaptation (ca) and the temperature sensitivity (ts) has not been fully identified. The consistent linkage of RNA1, RNA2 and NP genes during recombination at 25^{o} might be due to their essential role in replication at 25^{o}. Another possibility is that genes from the cold mutant may predominate simply because of greater efficiency of replication at 25^{o}. However, recent studies (20) using the same parental types but with recombination proceeding at 38^{o} temperature, which is nonpermissive for the cold mutant, recombinant clones were isolated with gene composition identical to the ones generated in recombination at 25^{o}. The recombinants derived at 38^{o} were cold-adapted, temperature-sensitive and attenuated in ferrets.

Thus, the segregation of the genes during recombination with the "Master strain" is independent of the temperature used during co-infection. If this hypothesis can be repeatedly validated, any recombination in the laboratory or in nature between this "Master strain" and a wild type influenza virus will favor emergence of a less virulent strain. The recent data of Cox, *et al.*, (15) have shown the existence of multiple lesions in the genes of the cold mutant donor strain. The findings will reinforce the previous assertion that recombinants deriving most of the genes from the A/AA/6/60-

H2N2 cold variant are less likely to regain their virulence than recombinants which have gene(s) derived from a mutant virus possessing a small number of single-step lesions. Recent studies in volunteers are in agreement with this view.

The role of the ts phenotype in the ca recombinant vaccine is not clear, since candidates with the ca marker and ts^+ were still attenuated. However, for now, it can be stated that the recommendation is still valid to have both markers (ca,ts) in cold recombinants.

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REFERENCES

1. Maassab, H.F. (1967). Nature (London) 213, 612.
2. Maassab, H.F., Francis, T., Jr., Davenport, F.D., Hennessy, A.V., Minuse, E. and Anderson, G. (1969) Bull. W.H.O. 41, 589.
3. Maassab, H.F., Cox, N.J., Murphy, B.R. and Kendal, A.P. (1977) Develop. Biol. Standard 39, 25.
4. Maassab, H.F., Spring, S.S., Kendal, A.P. and Monto, A.S. (1978) "Negative Strand Viruses.
5. Dubes, G.R. and Wenner, H.A. (1957) Virology 41, 275.
6. Kitayama, T., Togo, Y., Hornick, R.B., and Friedwald, W.T. (1973) Infec. Immunity 7, 119.
7. Beare, A.S., Maassab, H.F., Tyrrell, D.A.J., Slepuskin, A.N. and Hall, T.S. (1971) Bull. W.H.O. 44, 593.
8. Kendal, A.P., Noble, G.R. and Dowdle, W.R. (1977) Virology 82, 111.
9. Mills, J.V. and Chanock, R.M. (1971) J. Inf. Diseases 128, 145.
10. Michaels, R.H., Mahmud, M.I.A., Coup, A.J., Jennings, R. and Potter, C.W. (1978) J. Med. Virol. 2, 258.
11. Abou-Donia, H., Jennings, R. and Potter, C.W. (1980) Arch. of Virol. 65, 99.
12. Murphy, B.R., Sly, D.L., Hosier, N.T., London, W.T. and Chanock, R.M. (1980) Infec. and Immunity 28, 688.

13. Davenport, F.M., Hennessy, A.V., Maassab, H.F., Minuse, E., Clark, L.C., Abrams, G.D. and Mitchell, J.R. (1977) J. Infect. Dis. 136, 17.
14. Cox, N.J., Maassab, H.F. and Kendal, A.P. (1979) Virology 97, 190.
15. Cox, N.J., Kendal, A.P., Maassab, H.F. and Konnecke, I. (1981) Manuscript in preparation.
16. Murphy, B.R., Holly, H.P., Berquist, E.J., Levine, M.M., Spring, S.S., Maassab, H.F., Kendal, A.P. and Chanock, R.M. (1979) Infec. and Immunity 23, 253.
17. Murphy, B.R., Rennels, M.B., Douglas, R.G., Jr., Betts, R.F., Couch, R.B., Cate, T.R. Jr., Chanock, R.M., Kendal, A.P., Maassab, H.F., Suwanagool. S., Sotman, S.B., Cisneros, L.A., Anthony, W.C., Nalin, D.R. and Levine, M.M. (1980) Infec. and Immunity 29, 348.
18. Reeve, P., Gerendas, B., Moritz, A., Liehl, E., Kunz, C. Hofmann, H. and Maassab, H.F. (1980) J. Med. Virol. 6, 75.
19. Lazar, A., Okabe, N. and Wright, P.F. (1980) Infect. and Immunity 27, 862.
20. Maassab, H.F., Smitka, C.W., Donabedian, A.M., Monto, A.S., Cox, N.J. and Kendal A.P. (1981) In "Replication of Negative Strand Viruses" Elsevier, New Holland, (In Press)

GENETIC AND BIOCHEMICAL ANALYSIS OF THE A/ANN ARBOR/6/60 COLD-ADAPTED MUTANT

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ABSTRACT

Using a variety of techniques including recombinational analysis, oligonucleotide mapping of viral RNA and comparative polyacrylamide gel electrophoresis of double-stranded RNA hybrids or viral polypeptides, we have detected mutations in all eight segments of the RNA of the A/Ann Arbor/6/60 cold-adapted and temperature-sensitive mutant. These mutations do not appear to affect the synthesis of virus-specific proteins, all of which can be detected in cells infected at the restrictive temperature. The presence of multiple RNA mutations in the attenuated mutant A/Ann Arbor/6/60 and the reproducible transfer of the six nonglycoprotein genes to recombinants may aid the preparation of reproducibly attenuated and phenotypically stable live attenuated influenza vaccine strains.

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INTRODUCTION

The A/Ann Arbor/6/60(H2N2) cold-adapted (ca) and temperature-sensitive (ts) mutant is being used as a gene donor for the production, by reassortment, of attenuated live influenza vaccine strains. A number of such cold-adapted recombinants have now been tested in human volunteers and have been demonstrated to be infectious for susceptible young adults and children, but rarely cause unacceptable vaccine associated side effects (1,2,3,4). Concomitant with human volunteer vaccine studies utilizing these cold-adapted recombinants, we are conducting genetic and biochemical analysis of the A/Ann Arbor/6/60 mutant and its recombinants in order to determine the nature of the lesions acquired during its adaptation to growth at 25°C. Our aim is to examine the molecular basis for the temperature-sensitivity, cold-adaptation and attenuation properties of the A/Ann Arbor/6/60 mutant and to identify biochemical markers that may be useful in studying the stability of the virus during human infections.

RESULTS

Genetic Analysis of Recombinants of A/Ann Arbor/6/60 Mutant

Gene Composition of Recombinants Produced at 25°.

Previously described candidate ca vaccine strains produced by recombination at 25° between the A/Ann Arbor/6/60 mutant and a variety of H3N2 and H1N1 viruses have a highly restricted gene composition; 51% of them derive all non-glycoprotein genes from the ca parent, and all of them derive a set of three genes from the ca parent (5). Additional ca recombinants have recently been produced with contemporary H1N1 and H3N2 wt parents, A/California/10/78 and A/Beijing/2/79 respectively. The gene assignments for these recombinants made using a variety of conditions of polyacrylamide gel electrophoresis (PAGE) (5,6) are shown in Table 1 along with gene assignments for the earlier ca recombinants. Of these forty recombinants, 61% derive all non-glycoprotein genes from the A/Ann Arbor/6/60 mutant parent. All cold-adapted recombinants prepared at 25°C examined so far obtained RNAs 1, 3 and the nucleoprotein gene from the ca parent.

TABLE 1. Gene Composition of Recombinant Cold-adapted Influenza Viruses

Recombinants					Derivation of genes					
Expt.	Wild-type parent	Clone	HA	NA	RNA1	RNA2	RNA3	NP	M	NS
AA-CR 6	A/Queensland/6/72	0	W	W	A	A	A	A	A	A
AA-CR 12	A/Ann Arbor/9/73	0	W	W	A	A	A	A	A	A
AA-CR 13	A/Dunedin/4/73	5	W	W	A	W	A	A	A	A
		9	W	W	A	W	A	A	A	A
AA-CR 18	A/Scotland/840/74	0	W	W	A	A	A	A	A	W
		4	W	A	A	A	A	A	A	W
		5	W	A	A	A	A	A	A	W
		6	W	W	A	A	A	A	A	W
		7	W	W	A	A	A	A	A	W
AA-CR 19	A/Victoria/3/75	0	W	W	A	W	A	A	A	A
AA-CR 22	A/Victoria/3/75	1	W	W	A	W	A	A	A	A
		17	W	A	A	A	A	A	A	A
AA-CR 29	A/Alaska/6/77[a]	2	W	W	A	A	A	A	A	A
		17	W	W	A	A	A	A	A	A
AA-CR 31	A/Alaska/6/77[a]	2	W	W	A	A	A	A	A	A
		3	W	W	A	A	A	A	W	A
		4	W	A	A	A	A	A	A	A
		10	W	W	A	A	A	A	A	W
		12	W	W	A	A	A	A	A	W
		13	W	W	A	A	A	A	A	A
		16	W	A	A	A	A	A	A	A
		17	W	A	A	A	A	A	A	A
		19	W	W	A	A	A	A	A	A
		20	W	W	A	A	A	A	A	A
AA-CR 33	A/USSR/90/77	1	W	W	A	A	A	A	A	A
		2	W	W	A	A	A	A	A	A
AA-CR 35	A/Hong Kong/123/77[a]	2	W	W	A	A	A	A	A	A
AA-CR 36	A/Hong Kong/123/77[a]	1	W	W	A	A	A	A	A	A
		2	W	W	A	A	A	A	A	A
		3	W	W	A	A	A	A	A	A
		4	W	A	A	A	A	A	A	A
		5	W	W	A	A	A	A	A	A
		7	W	W	A	A	A	A	A	A
		9	W	W	A	A	A	A	A	A
		10	W	W	A	A	A	A	A	A
AA-CR 37	A/California/10/78	1	W	W	A	A	A	A	A	A
AA-CR 44	A/Beijing/2/79[a]	6	W	W	A	A	A	A	A	A
		11	W	W	A	A	A	A	A	W
		16	W	W	A	A	A	A	A	A
		20	A	W	A	A	A	A	A	A

W indicates gene derived from wild-type parent.

A indicates gene derived from mutant parent.

[a]Adapted to growth in primary bovine kidney cells and cloned at 39° in these cells.

Gene Composition of Recombinants Produced at 33° or 39°. The predominance of mutant genes in recombinants of the A/Ann Arbor/6/60 virus produced at 25° prevented us

Table 2. Properties of Recombinants Derived from Mixed Infections of A/Ann Arbor/6/60 Mutant and WSN Mutants or A/Ann Arbor/9/73 Wild-type.

Recombinant clone designation	Gene Derivation[c] 1	2	3	4 (HA)	5 (NA)	6 (NP)	7 (M)	8 (NS)	EOP PFU 39°/ PFU 33°	
Ia_3[a]	A	A	W	W	A	A	W	A	12%	(ts^+)
Iu_2[a]	A	W	W	W	A	W	W	W	67%	(ts^+)
Im_3[a]	A	W	W	W	A	A	W	A	25%	(ts^+)
IIg_1[a]	A	W	A	A	A	A	W	A	25%	(ts^+)
IIh_2[a]	W	A	A	A	A	W	W	A	40%	(ts^+)
Vh_5[a]	W	W	A	W	W	A	W	W	50%	(ts^+)
$VIIb_4$[a]	W	W	W	A	A	W	A	W	100%	(ts^+)
$VIIc_1$[a]	W	A	W	W	A	W	A	A	25%	(ts^+)
S_{32}[b]	W	W	A	A	A	A	A	A	51%	(ts^+)
S_{34}[b]	W	A	A	A	A	A	A	A	19%	(ts^+)
S_1[b]	A	W	W	W	A	W	A	A	<0.001%	(ts)
S_{10}[b]	A	A	A	A	A	A	A	W	<0.001%	(ts)
M_4[b]	A	W	A	W	W	A	A	A	<0.002%	(ts)
No. ts^+ recombinants with A/Ann Arbor/6/60 gene	4/10	4/10	5/10	5/10	6/10	9/10	4/10	7/10		
No. ts recombinants with A/Ann Arbor/6/60 gene	3/3	1/3	2/3	1/3	2/3	2/3	3/3	2/3		

[a]Clones derived by recombination at 39° of the A/Ann Arbor/6/60 mutant and WSN ts mutants.

[b]Clones derived by recombination at 33° of the A/Ann Arbor/6/60 mutant and the A/Ann Arbor/9/73 wild-type.

[c]A indicates the gene was derived from the A/Ann Arbor/6/60 mutant; W indicates the gene was derived from the WNS mutant for clones designated *a* or the A/Ann Arbor/9/73 wild-type for clones designated as *b*.

from identifying the gene or genes carrying the ts and ca mutations. In the hope of obtaining more genetic diversity among recombinants, we produced recombinants at 33° and at 39° using the A/Ann Arbor/9/73 H3N2 wt virus or selected WSN ts mutants, respectively, as the second parent (7). As shown in Table 2, recombinants which possessed fewer mutant genes were obtained; for example, recombinant $VIIb_4$ possesses only three genes from the mutant virus. Furthermore, in the recombination experiment performed at 33° both ts and ts^+ viruses were isolated, and we were able to segregate the ts and ca phenotypes. Thus we could begin to analyze the genetic basis for these properties. Our analysis indicates that each A/Ann Arbor/6/60 mutant gene is present in several of the ts^+ recombinants (Table 2). Several ts^+ recombinants contain predominantly A/Ann Arbor/6/60 genes. For example, ts^+ recombinants IIg_1, and S_1 lack only RNAs 2 and 7 or RNA 1 of the A/Ann Arbor/6/60 respectively, suggesting the involvement of one or more of these genes in the ts phenotype. However, by themselves these genes do not confer temperature sensitivity since ts^+ recombinants $VIIb_4$ and $VIIc_1$ both possess A/Ann Arbor/6/60 RNA 7, A/Ann Arbor/6/60 RNA 2 was present in three ts^+ recombinants, and A/Ann Arbor/6/60 RNA 1 was present in four viruses. This implies that no single mutant gene by itself confers temperature sensitivity. However, the combination of RNAs 1 and 7, RNAs 3 and 7 or RNAs 5 and 7, were missing from all ts^+ recombinants. Examination of the ts recombinants S_1, S_{10} and M_4 revealed that, of these combinations, only RNAs 1 and 7 were present in all cases. The results of this analysis permits us to conclude that the presence of both RNAs 1 and 7 of the A/Ann Arbor/6/60 mutant is necessary to confer the ts property. The alternative hypothesis that a high frequency of suppressor mutations or reversions occurs during recombination not only at 39°, but also at 33° in tissue culture, seems unlikely since such conditions would not be expected to exert selective pressure for the generation of such mutations.

Biochemical Evidence for Lesions in RNA of A/Ann Arbor/6/60 Mutant

RNA Oligonucleotide Analysis of RNA of Mutant and WT A/Ann Arbor/6/60. The complexity of the above genetic analysis prompted us to seek direct biochemical evidence for RNA mutations in the genes thought to confer the ts and ca properties, as well as in other mutant genes. Total virion

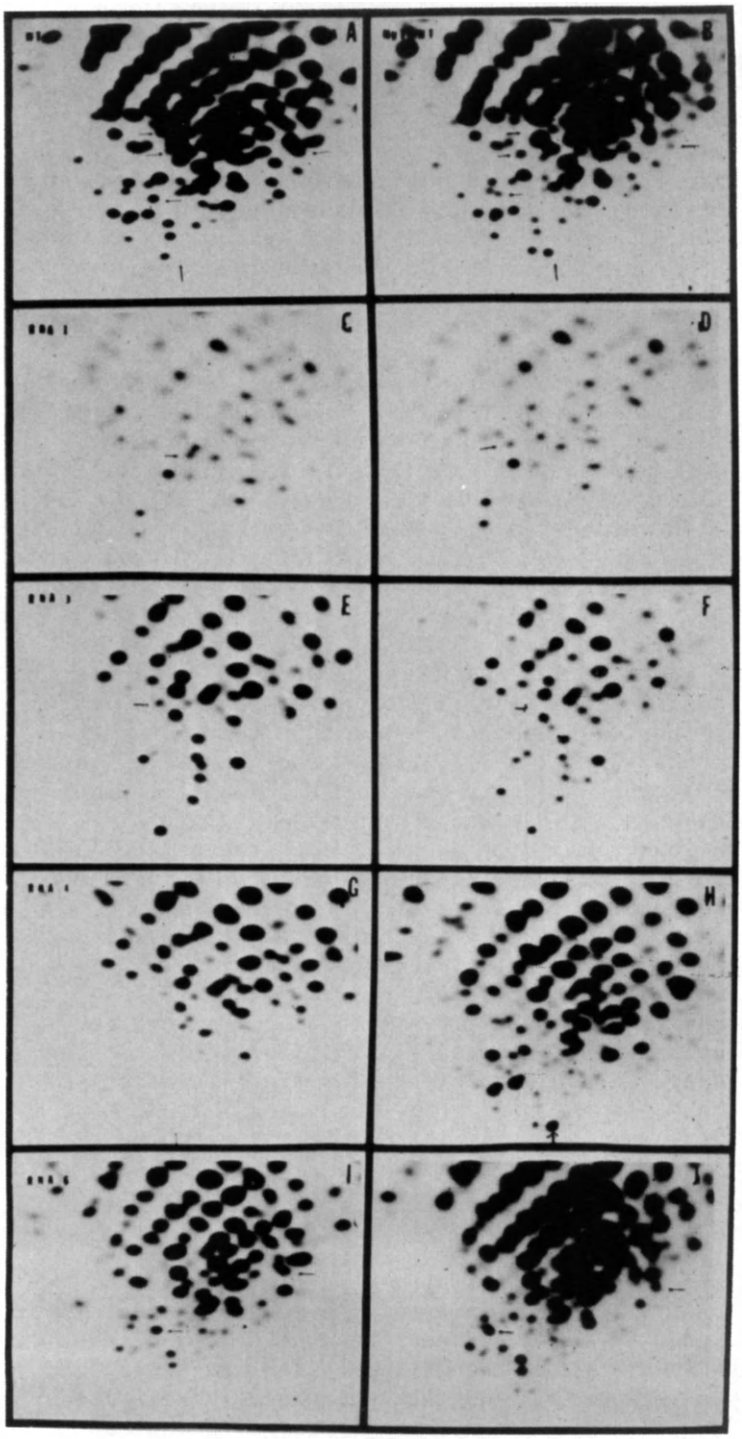

Figure 1

FIGURE 1. Oligonucleotide maps of A/Ann Arbor/6/60 wt and mutant RNAs. A. Total virion RNA of A/Ann Arbor/6/60 wt; B. Total virion RNA of A/Ann Arbor/6/60 mutant; C. RNA 1 of wt; D. RNA 1 of mutant; E. RNA 3 of wt; F. RNA 3 of mutant; G. RNA 4 of wt; H. RNA 4 of mutant; I. RNA 6 of wt; J. RNA 6 of mutant.

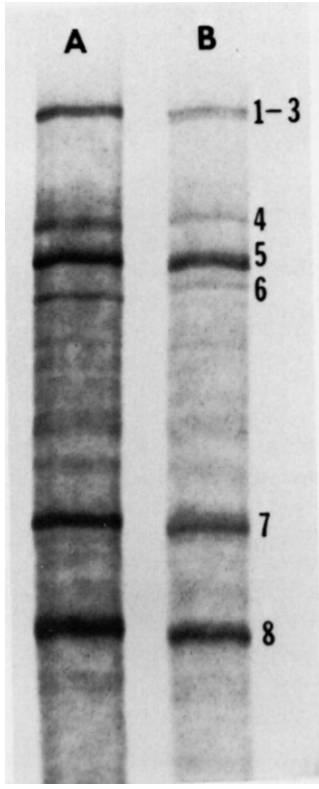

FIGURE 2

FIGURE 2. Analysis of homologous and heterologous ds RNAs. A. The homologous RNA hybrids obtained with cRNA from mutant virus infected cells and vRNA from mutant virus. B. The heterologous RNA hybrids obtained with cRNA from mutant virus and vRNA from wt virus.

RNA was prepared for wt and mutant viruses, the RNA was digested with T_1 ribonuclease, labeled using $\gamma^{32}P$ ATP and polynucleotide kinase and analyazed by two-dimensional electrophoresis (8,9,10). The T_1 maps showed a minimum of five differences observed in oligonucleotides of the mutant virus as compared with wt virus (Fig. 1A and 1B) if it is assumed that the spot changes that appeared to occur as pairs resulted from a single base change in each case. In order to assign the oligonucleotide changes to individual genes, oligonucleotide maps of the isolated RNA segments from mutant and wt viruses were compared. RNA 1 of the mutant virus exhibited a one oligonucleotide spot difference from the corresponding wt RNA segment (Fig. 1C and 1D). Oligonucleotide differences were also found in RNA 3 (one difference)(Fig. 1E and 1F), RNA 4 (Fig. 1G and 1H) and the nucleoprotein gene (Fig. 1I and 1J). No oligonucleotide changes were detected for RNA segments 2, 5 (NA), 7 (M) or 8 (NS).

Analysis of S_1 Nuclease Treated RNA-RNA Hybrids. T_1 oligonucleotide mapping procedures yielded no evidence for mutations in RNA 7 of the mutant virus though other evidence indicated that changes would be expected. It has been reported that in some instances single base changes can be detected by PAGE analysis of S_1 nuclease digested RNA-RNA hybrids formed between cRNA transcripts extracted from cells infected in the presence of cycloheximide and an excess of virion RNA (11,12). Analysis of double-stranded (ds) hybrids on a 7.5% polyacrylamide gel after treatment of hybridization mixtures with S_1 nuclease at a concentration of 1000 units/mg of RNA/ml, demonstrated altered electrophoretic mobilities for the heterologous RNAs 4, 5, 6, 7 and 8 (Fig. 2B) as compared to the homologous RNAs obtained from the A/Ann Arbor/6/60 mutant (Fig. 2A).

Analysis of Viral Protein Synthesis

Comparison of Polypeptides Synthesized at Permissive and Nonpermissive Temperatures. In order to determine if virus-specific proteins are synthesized at nonpermissive temperatures (25^o for wt virus and 39^o for mutant virus) Madin-Darby canine kidney (MDCK) cells or primary chick kidney (pCK) cells in 25 cm^2 T-flasks were infected with 1.0 ml of virus inoculum at 20 - 100 PFU/cell (13). [S^{35}] methionine was added at various times post infection (p.i.) for 15 min. Cells were then harvested, dissociated and analyzed by PAGE on 25% polyacrylamide gels as described

previously (14). Virus growth in MDCK cells at permissive and nonpermissive temperatures was determined in parallel by plaque titration of tissue culture fluids collected at selected intervals p.i. as described previously (15). As shown in Figure 3B, though the detection of protein synthesis is delayed and the level of protein synthesis is lower at 25° as compared with 33°, proteins P_1, P_2, P_3, HA, NA, NP, M and NS, of both A/Ann Arbor/6/60 mutant and wt are synthesized. On the other hand, production of infectious wt virus was reduced by 2-3 logs (Table 3). At 39°, the A/Ann Arbor/6/60 mutant virus does not produce infectious virus progeny (Table 3); however, an abundance of all virus-specified proteins was detected (Figure 3A).

TABLE 3. Growth of Mutant and Wild-type A/Ann Arbor/6/60 Viruses in MDCK Cells at Permissive and Restrictive Temperatures

	Infectivity titrations (PFU/ml) in MDCK cells at					
	25°		33°		39°	
Hours p.i.	Mutant	Wild-type	Mutant	Wild-type	Mutant	Wild-type
12	0	0	7×10^5	5×10^5	0	8×10^4
24	0	0	2×10^7	1×10^7	0	2×10^5
72	4×10^5	2×10^3	ND*	ND	ND	ND
132	1×10^6	5×10^3	ND	ND	ND	ND

*ND indicates not determined due to cell death.

Figures 3A and 3B demonstrate clearly that the 'P_2' protein of the A/Ann arbor/6/60 mutant virus migrates more slowly than that of wt virus. Other virus-specific proteins migrated similarly in this gel system. By analyzing virus-specific proteins in cells infected with recombinants S_1, S_{32} and M_4 (Table 3) we were able to determine that RNA 3 codes for the altered polypeptide 'P_2'. These results extend earlier studies of polypeptide synthesis in mutant and wt infected cells (18) by demonstrating a lack of leakiness at high m.o.i. and by demonstrating a change in electrophoretic migration of the polymerase protein 'P_2' which is coded for by RNA segment 3.

FIGURE 3

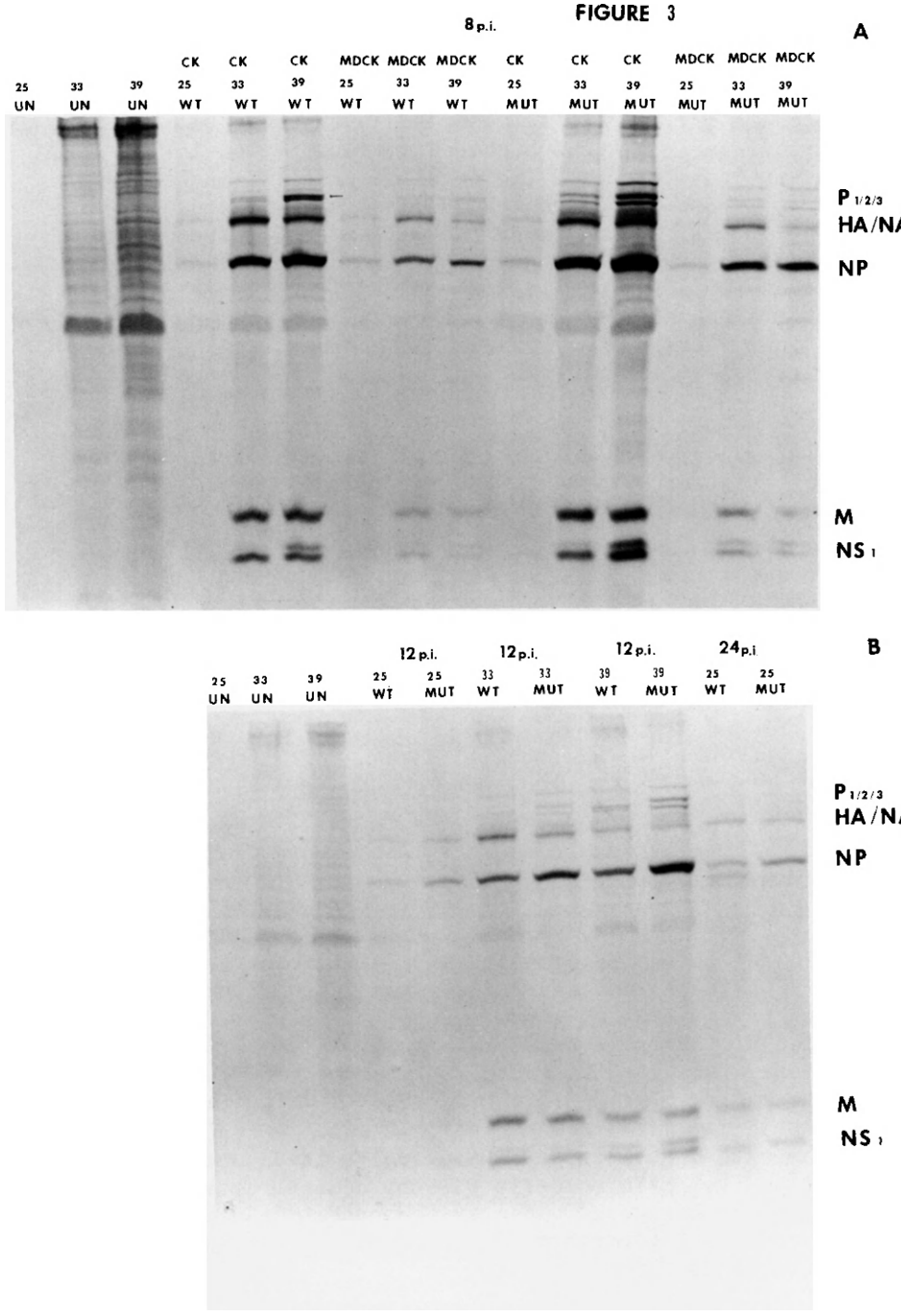
A
8 p.i.
CK CK CK MDCK MDCK MDCK CK CK CK MDCK MDCK MDCK
25 33 39 25 33 39 25 33 39 25 33 39 25 33 39 25 33 39
UN UN UN WT WT WT WT WT WT MUT MUT MUT MUT MUT MUT
P 1/2/3
HA/NA
NP
M
NS 1
B
12 p.i. 12 p.i. 12 p.i. 24 p.i.
25 33 39 25 25 33 33 39 39 25 25
UN UN UN WT MUT WT MUT WT MUT WT MUT
P 1/2/3
HA/NA
NP
M
NS 1

FIGURE 3. Virus-specific protein synthesis in A/Ann Arbor/6/60 mutant and wt infected cells. A. Protein synthesis 8 h p.i. in MDCK or CK cells at 25°, 33° or 39°. B. Protein synthesis 12 and 24 h p.i. in CK cells at 25°, 33° or 39°.

DISCUSSION

Here we have described the restricted genetic composition of the candidate vaccine strains produced by recombination at 25° to date. Additional recombinants produced at 33° or 39° have also been genotyped and allow us to conclude that the ts property of the A/Ann Arbor/6/60 mutant requires the presence of RNAs 1 and 7. In addition, we have described comparative biochemical analysis of the mutant and of the wt parent from which it was derived demonstrating mutations in all eight genes of the ca variant (Table 4). Thus far we have detected changes only in gene products P_2 and HA (18). In order to determine what effect, if any, these mutations might have on virus replication, we analyzed virus specific protein synthesis of the mutant and wt A/Ann Arbor/6/60 viruses at permissive and restrictive temperatures. An abundance of all virus specific proteins were synthesized at restrictive temperatures (39° for the mutant), however, no infectious virus was produced. These experiments indicated that the ts defect of the mutant probably does not operate at the level of cRNA transcription or protein translation.

Live influenza A vaccine strains deriving the six nonglycoprotein genes from the ca A/Ann Arbor/6/60 are usually attenuated and immunogenic. The question of genetic stability is of equal importance to attenuation and efficacy in the development of live-attenuated vaccine strains. It has been shown that a ts influenza vaccine strain when administered to totally susceptible individuals having no detectable antibody to the viral hemagglutinin or neuraminidase, underwent genetic modifications including suppressor and intragenic mutations (16). These findings have led to speculation that since influenza virus undergoes mutation with a high frequency when replicating in a completely susceptible host, efforts to develop stably attenuated mutants for use in vaccination may be futile. In contrast to findings with the ts viruses, however, true revertants (i.e. viruses that are ts^+ and ca^+) of cold recombinant vaccine strains have not yet been isolated from human volunteers or animals. Only viruses with a modified

Table 4. Summary of Comparative Analysis of A/Ann Arbor/6/60 Mutant and Wild=type for Evidence of Genetic Mutation

Gene	Mutation	Methods(s) detected	Possible functional effect
RNA1	✓	PAGE of virion RNA. Oligonucleotide mapping (one spot). Recombinational analysis.	Synergistic role in ts with RNA 7. Interference in complementation with NIH group I mutant.[a]
RNA 2	✓	Recombinational analysis.	*In vitro* RNA transcriptase activity.[b] Fails to rescue WSN group III mutant.[c]
RNA 3	✓	PAGE of intracellular proteins. Oligonucleotide mapping (one spot). Recombinational analysis.	Not known.
RNA 4 (HA)	✓	Antigenic analysis. Oligonucleotide mapping (one spot). PAGE of intracellular proteins. PAGE of S_1 nuclease-treated RNA-RNA hybrids.	Not known.
RNA 5 (NA)	✓	Page of S_1 nuclease-treated RNA-RNA hybrids.	Not known.
RNA 6 (NP)	✓	Oligonucleotide mapping (two spots). PAGE of S_1 nuclease-treated RNA-RNA hybrids	Not known
RNA 7 (M)	✓	PAGE of S_1 nuclease-treated RNA-RNA hybrids. Recombinational analysis	Synergistic role in ts with RNA 1.
RNA 8 (NS)	✓	PAGE of S_1 nuclease-treated RNA-RNA hybrids.	Not known.

[a] References 7 and 18.

[b] Reference 6.

[c] Reference 7.

ca property or viruses that had partially reverted in ts or ca phenotype have been observed. Furthermore, reversion of attenuation to virulence may be independent from reversion of the phenotypic markers of cold adaptation or temperature sensitivity because it is not known that these markers are relevant to attenuation of ca A/Ann Arbor/6/60. The A/Ann Arbor/6/60 mutant virus was derived by sequential passage at consecutively lower temperatures in pCK cells until a variant virus that replicated efficiently at 25° emerged. The presence of multiple mutations in the genes of c.a. A/Ann Arbor/6/60 provides a basis for explaining why reversion to wild-type by this virus (or by recombinants prepared as live vaccine strains that contain all of its non-glycoprotein genes) has occurred less frequently than reversion of vaccine candidate possessing only one or two chemically induced ts mutations.

ACKNOWLEDGEMENTS

We thank Judy Galphin, Connie Carmack, Eve Bingham and Donna Sasso for excellent technical assistance. This work was supported in part by National Institutes of Health Contract No. 1-Y01-A1-80001.

REFERENCES

1. Davenport, F. M., Hennessy, A. V., Maassab, H. F., Minuse, E., Clark, L. C., Abrams, G. D., and Mitchell, J. R. (1977). J. Infect. Dis. 136, 17.
2. Hrabar, A., Vodpija, I., Andre, F. E., Mitchell, J. R., Maassab, H. F., Hennessy, A. V., and Davenport, F. M. (1977). Dev. Biol. Stand. 39, 53.
3. Murphy, B. R., Holley, H. P. Jr., Berquist, E. J., Levine, M. M., Spring, S. B., Maassab, H. F., Kendal, A. P., and Chanock, R. M. (1979). Infect. Immun. 23, 253.
4. Murphy, B. R., Rennels, M. B., Douglas, R. G., Betts, R. F., Couch, R. B., Cate, T. R., Chanock, R. M., Kendal, A. P., Maassab, H. F., Suwanagool, S., Sotman, S. B., Cisneros, L. A., Anthony, W. C., Nalen, D. R., and Levine, M. M. (1980). Infect. Immun. 29, 348.
5. Cox, N. J., Kendal, A. P., and Maassab, H. F. (1979). Virology 97, 190.

6. Kendal, A. P., Cox, N. J., Galphin, J. C., and Maassab, H. F. (1979). J. Gen. Virol. 44, 443.
7. Cox, N. J., Kendal, A. P., Maassab, H. F., Scholtissek, C., and Spring, S. B. (1981). In "The Replication of Negative Strand Viruses" (D. Bishop and R. Compans, eds.) Elsevier North-Holland, Inc., New York, in press.
8. DeWachter, R., and Fiers, W. (1972). Anal. Biochem. 49, 184.
9. Frisby, D. (1977). Nucleic Acids Res. 4, 2975.
10. Pederson, F. S., and Haseltine, W. S. (1980). Meth. Enzymology 65, 680.
11. Hay, A. J., Lomniczi, B., Bellamy, A. R., and Skehel, J. J. (1977). Virology 83, 337.
12. Hay, A. J., Skehel, J. J., and Webster, R. G. (1979). J. Gen. Virol. 45, 245.
13. Kendal, A. P., Cox, N. J., Murphy, B. R., Spring, S. B., and Maassab, H. F. (1977). J. Gen. Virol. 37, 145.
14. Konnecke, I., Boschek, B., and Scholtissek, C. (1981). Virology, in press.
15. Cox, N. J., O'Neill, M. C., and Kendal, A. P. (1977). J. Gen. Virol. 37, 161.
16. Murphy, B. R., Tolpin, M. D., Massicot, J. G., Kim, H. Y., Parrott, R. H., and Chanock, R. M. (1980). Annals of the New York Acad. of Sci. 354, 172.
17. Maassab, H. F. (1967). Nature (London) 213, 612.
18. Kendal, A. P., Kiley, M. P., and Maassab, H. F. (1973). J. Virol. 12, 1503.

ANTI-INFLUENZA HEMAGGLUTININ RESPONSE INDUCED WITH SYNTHETIC ANTIGEN

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For many years we have been interested (Arnon, 1972; Sela 1975; Arnon, 1979) in the possibility that vaccines used today might be replaced sometime in the future with antigens containing unique synthetic determinants, capable of provoking antibodies efficiently neutralizing disease inducing agents including viruses. We know that only a fraction of antibodies provoked by a complex macromolecular immunogen which possesses biological activity is directly involved in the neutralization of that activity. Thus, the administration of many unnecessary determinants, not to speak about irrelevant associated antigens, could be avoided.

When the chemical structure of a protein antigenic determinant is known, it is possible to synthesize it, if necessary attach it to a carrier (synthetic or a protein), and use the resulting conjugate for elicitation of antibodies reactive with the intact protein. Antibodies provoked in this manner towards the synthetic peptide corresponding to the "loop" region of the enzyme lysozyme were shown to be directed to a conformation-dependent determinant, and they react with the intact native protein (Arnon et al., 1971). In another study we have shown that a synthetic peptide corresponding to the N-terminal region of carcinoembryonic antigen (CEA) elicited in rabbits antibodies recognizing the intact CEA molecule (Arnon et al., 1976). These antibodies served in a viroimmunoassay for detection of CEA in sera of cancer patients (Arnon et al., 1977). It should be, therefore, feasible to apply a similar approach for components of viruses or bacteria.

As a first model system for testing this possibility, we chose the coliphage MS-2, an RNA-containing virus with icosahedral symmetry. The viral capsid contains 180 identical coat protein subunits with a molecular weight of 13,700 daltons each. Antibodies against the coat protein are as efficient as anti-phage sera in neutralizing the MS-2 phage. The coat protein consists of a single polypeptide chain with 129 amino acid residues, with a known sequence. We have cleaved this coat protein with cyanogen bromide and obtained a mixture of two small peptides, P_2 and P_3 of about 20 amino

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acids each, which was found capable of inhibiting the neutralization of phage by antiserum to the whole phage (Fig. 1). In order to identify which of the two is the inhibitory active material the peptides corresponding to P_2 and P_3 were synthesized (Langbeheim et al., 1976). The results indicated that the synthetic P_3, corresponding to the carboxy-terminal 21 amino acid residues in the sequence of the coat protein, had no capacity to interfere with the neutralization of MS-2 (Fig. 1), nor did its macromolecular conjugate with multichain poly-DL-alanine (denoted P_3-A--L) elicit neutralizing antibodies, when used for immunization of rabbits in complete Freund's adjuvant (Fig. 2). On the other hand, the synthetic P_2 peptide was very efficient in inhibiting the inactivation of the phage by the antiserum against the phage (Fig. 1). Furthermore, a synthetic antigen prepared by attachment of P_2 covalently to multichain poly-DL-alanine induced antiserum in rabbits, when administered in complete Freund's adjuvant, that was capable of neutralizing MS-2 activity almost as efficiently as the antiserum prepared in rabbits against the intact coat protein (Fig. 2). This inactivation was specific, because it could, in turn, be totally inhibited by the P_2 peptide.

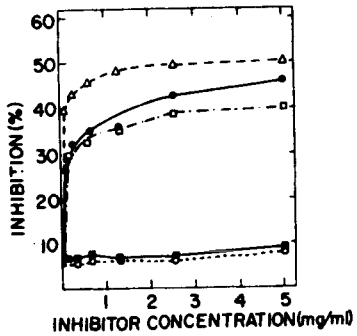

Fig. 1. Inhibition of MS-2 phage neutralization by antiserum against phage (dilution 3 x 10^{-4}) with synthetic peptides: intact coat protein (Δ---Δ), mixture of the native peptides P_2 and P_3 (●—●), synthetic peptide P_2 (□ ····· □), synthetic peptide P_3 (■ —— ■), phosphate buffer control (o---o). From Langbeheim et al. (1976).

This study provided the first reported evidence that a synthetic peptide corresponding to a region which is involved in viral neutralization can be utilized for eliciting antiviral activity. It is logical to assume that the P_2 peptide assumes in P_2-A--L a steric conformation close to the one which this peptide region has in the intact coat protein, and

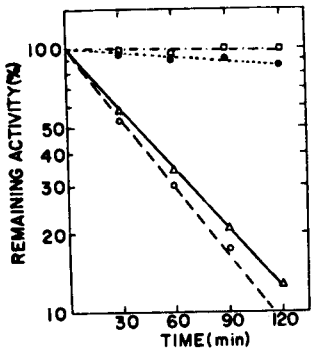

Fig. 2. Neutralization of MS-2 phage by anti-conjugate serum. Antiserum against coat protein (dilution 10^{-3}) (o---o), antiserum against P_2-A--L (dilution 3×10^{-3}) (Δ——Δ), antiserum against P_3-A--L (dilution 3×10^{-3}) (•---•), and normal serum (dilution 10^{-3}) (□ ····· □). From Langbeheim et al. (1976).

that this conformation is conserved also in the intact phage.

In a more recent study (Arnon et al., 1980) we demonstrated that when the synthetic adjuvant muramyl-dipeptide (MDP) was linked to the P_2-A--L conjugate, it yielded a conjugate which elicited in rabbits, when administered in phosphate buffered saline, almost as good anti-MS-2 response as did the immunization in complete Freund's adjuvant (Fig. 3).

These results illustrated the challenges and the advantages of the synthetic approach to preparation of vaccines and demonstrated that it is feasible to design a completely synthetic molecule, which contains built-in adjuvanticity and consequently, even in aqueous solution, can provoke anti-viral immune response.

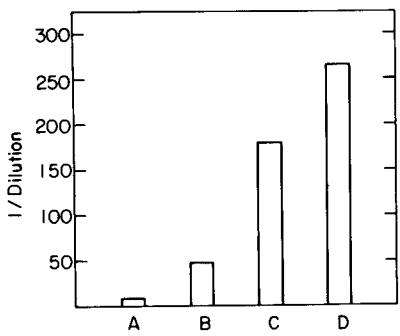

Fig. 3. Adjuvant effect of MDP on the anti-viral activity of P_2-A--L conjugate. The bars designate the reciprocal dilution effecting 50% inactivation of the virus of antisera obtained by immunization of rabbits with: A: P_2-A--L in phosphate buffered saline (PBS); B: P_2-A--L+MDP in PBS; C: MDP-P_2-A--L conjugate in PBS; D: P_2-A--L in CFA.

It seemed of interest to test this chemical approach to vaccination in an animal virus. The influenza virus constitutes a very suitable model for an animal virus for this purpose for the following reasons: 1) Detailed information is available on the structure, function and antigenic components of this virus, as well as on its serological specificities and genetic variations. 2) Various reliable assays of the virus are available, such as a radioimmunoassay, hemagglutination of chicken erythrocytes (Fazekas De St. Growth et al., 1966), *in vitro* plaque assay (Kilbourne, 1969), as well as *in vivo* infectivity in mice (Webster and Askonas, 1980), which provide adequate means for evaluating the effect of the immune response on the different viral functions. 3) Sufficient information is available on the amino acid sequence of the influenza hemagglutinin (Laver and Air, 1979), and on its immunochemical properties (Jackson et al., 1979), to allow the synthesis of peptide fragment(s) which might carry some of its immunological activity.

Synthesis and characterization of the hemagglutinin peptide. The synthetic peptide used in this study corresponds to the region 91-108 in the amino acid sequence of the HA heavy chain of H_3N_2 strains. This sequence is part of the HA_1CN_1 fragment, which was shown by Jackson et al. (1979) to be immunologically active. This peptide is common for at least nine H_3N_2 strains, and the rationale for choosing it was the following: Due to its large size the HA_1CN_1 fragment as such is not readily amenable to chemical synthesis, nor was this fragment available in sufficient quantities to allow the preparation and screening of smaller immunologically active fragment(s). Since at the time this investigation was initiated, about one and a half years ago, the three-dimensional structure of the influenza hemagglutinin was not yet known, the decision on the peptide to be synthesized was made according to the available amino acid sequence (Laver et al., 1980), which was very kindly provided to us by Drs. C.W. Ward and W.G. Laver, prior to publication, as well as the predicted folded structures of the hemagglutinin molecule. The prediction of this structure was derived from a computer analysis based on the amino acid sequence, using the algorithms developed for this purpose by Dr. Christian Sander from the Department of Chemical Physics of this Institute.

The synthesized peptide, which according to the predicted structure should have comprised a β-bend folded region, consisted of the residues 91-108 of HA_1. This is a region common to at least 9 H_3N_2 strains of the A subtype (Laver et al., 1980). It contains 2 proline residues, which are known to

play a crucial role in the spatial conformation of proteins (Arnon et al., 1974) and their antigenic specificity (Teicher et al., 1975). It also contains 3 residues of tyrosine, an amino acid with a putative contribution towards the immunogenic capacity (Arnon and Sela, 1960). This peptide was thus anticipated to be immunogenic, hopefully retaining some distinct conformational features of the native protein. It is of interest that this region was indeed reported to possess a folded "corner" in the three dimensional structure of the influenza hemagglutinin, as reported recently by Wiley et al. (1981). Consequently, although not designated by these authors as one of the antigenic sites, it could readily be visualized as an exposed region which has an immunological imprint. We have, therefore, anticipated that it would elicit anti-influenza antibodies.

The synthesized peptide, denoted $HA_1(I)$ served for conjugation to three different carriers, two synthetic ones, namely multichain poly-DL-alanine and multichain poly-L-proline, and one natural carrier, purified tetanus toxoid (TT). Rabbits were immunized with all three carriers, and the Ig fraction of the resulting antisera were evaluated for the presence of anti-peptide antibodies. These were demonstrated in anti-$HA_1(I)$-TT serum, both by gel diffusion in agar, and by radioimmunoassay on antigen-coated microtiter plates, using the peptide as antigen (Fig. 4). The reaction can be demonstrated up to a dilution of 1:10,000.

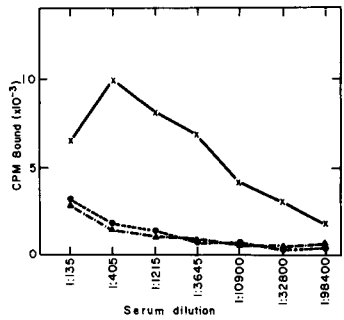

Fig. 4. Solid phase radioimmunoassay of anti-$HA_1(I)$-TT serum with $HA_1(I)$. (x—— x): reaction with antigen coated plates; (●——●): reaction with uncoated plates; (▲---▲): controls with preimmune serum.

Anti-influenza immune response induced by the synthetic conjugates. The presence of anti-influenza antibodies in the serum obtained from rabbits after immunization with the $HA_1(I)$-TT conjugate was demonstrated by the cross-reaction with either the viral antigen (denatured virus) or the intact live virus of different strains, using both the radioimmunoassay and the hemagglutination inhibition test. In the radioimmunoassay there is a high cross-reaction of the antibodies with the intact virus of the serum resistant A/Tex/77 strain. Cross-reactivity was obtained with two other A strains, namely A/Mem/72 and A/Vic/75 which gave higher binding to the immune serum as compared to the pre-immune serum (not shown). The B/Vic strain shows also some cross-reaction with the antiserum, but to a lower extent than that of the A strains (Fig. 5).

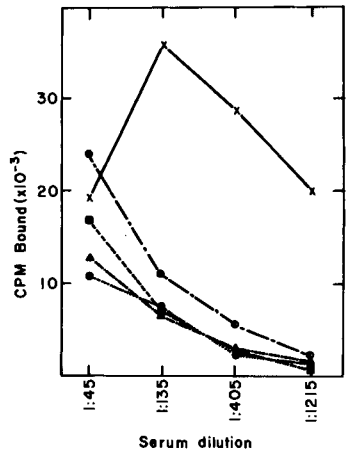

Fig. 5 Solid phase radioimmunoassay of rabbit anti-$HA_1(I)$-TT with the intact virus A/Tex/77 (x—— x), B/Vic (o —· — · — o) or no antigen (●---●); control of preimmune serum with A/Tex/77 (▲———-———▲) or B/Vic (■ ---- ■).

The results of the hemagglutination inhibition test by the anti $HA_1(I)$-TT are given in Fig. 6. As shown, the hemagglutination of chicken erythrocyte by the virus was partially inhibited by both the rabbit antiserum and by the sera of mice of several strains which were immunized with the peptide conjugate.

In vitro neutralization of the virus by the anti-peptide serum. The neutralization of the virus was tested by the capacity of the rabbit anti $HA_1(I)$-TT Ig to inhibit the virus plaque formation in tissue culture *in vitro*. The virus used in this assay was the serum resistant A/Texas strain. The results demonstrating the neutralization of the virus, at different dilutions, by different concentration of the Ig fraction of the immune serum, are given in Table 1. As shown, at all virus dilutions tested, the antibodies caused

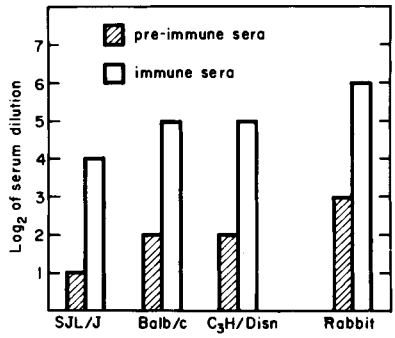

Fig. 6. Inhibition hemagglutination of chicken erythrocytes by A/Vic antigen obtained with sera of rabbit and three different mouse strains immunized with $HA_1(I)$-TT, as compared with the hemagglutination inhibition by the respective pre-immune sera.

inhibition of virus plaque formation, which is dependent on the antibody concentration and apparently on virus dilution as well. With the lowest virus concentration the extent of inhibition is higher. The antiserum is effective up to a dilution of 1:32, a value which is consistent with the results of the hemagglutination inhibition.

Table 1. *In vitro* neutralization of virus by antiserum (Plaque inhibition)

Dilution of allantoic fluid	No. of plaques/ plate	% Inhibition of plaque formation at different serum dilutions			
		1:16	1:32	1:64	1:128
10^{-6}	113	24	20		
3×10^{-7}	35	55	38	31	0
10^{-7}	13	65	67		

Protection of mice against infection. The mouse strain C3H/DiSn was selected for checking the potential of the synthetic conjugate to provide protection against viral challenge. The preliminary results of such experiments, demonstrated in Table 2, indicate that immunization of the mice with the $HA_1(I)$-TT conjugate, resulted in their effective protection against infection with the A/Texas mouse-adapted influenza virus. The rate of infection was determined by

evaluating the number of infective virus particles that can be recovered from the lungs of the immunized and non-immunized mice. The protective effect is demonstrated by the difference in the incidence of the infection in the mice, as well as by the lower egg infective dose of the lung homogenates of the immunized mice as compared to control groups, which were either untreated, or immunized with the tetanus toxoid alone, prior to infection. It should be mentioned that no protection was observed in an additional experiment in which a higher infective dose (a 10^{-6} dilution) of the virus has been used. The effect of immunization is stronger when the dose of the virus is lower, a phenomenon similar to that observed in the *in vitro* assay (Table 1). However, evidence is presented for an effective anti-viral immunity which is elicited by a synthetic material.

Table 2. Protection of mice against infection[a]

Group	Dilution of virus used for infection	Incidence of infection[b]		EID[c]
		10^{-1} dilution into eggs	10^{-2} dilution into eggs	
Exp. 1	10^{-8}			
Control (untreated)		4/4	3/4	$10^{-3.5}$
Control (toxoid)		4/4	3/4	10^{-4}
Vaccinated		2/4	1/4	$10^{-1.25}$
Exp. 2	10^{-7}			
Control (untreated)		3/5	2/5	$10^{-2.2}$
Vaccinated		2/10	1/10	$10^{-0.7}$

a) Mice were immunized in CFA and boosted at 4 weeks. Infection was performed 5 days after the booster injection, and 3 days later lungs were removed for evaluation.
b) Number of mice per group in which virus was present in a 10% lung homogenate.
c) Egg-infective dose - lowest dilution of lung homogenate which is still infective in eggs (average values for all the mice in each group).

A characteristic of the influenza system which plays a paramount role in the immune response towards it, is the tremendous genetic variation among influenza viruses and its reflection in their antigenic differences. With the extensive data accumulated recently by both sequence analysis and the use of monoclonal anti-influenza antibodies, several particular amino acid exchanges that may be correlated with the antigenic "shifts" and "drifts" have been identified (Laver et al., 1979; Wiley et al., 1981). With this available information on "variable" and "constant" regions in the hemagglutinin molecule it should be feasible, by the use of the appropriately designed synthetic antigens, to direct the immune response against one or the other of these two molecular entities. As already indicated, the peptide used in this study comprises a part of a preserved sequence, which is present in at least 9 different H_3N_2 strains of subtype A. Hence, on principle, the antibodies it elicits should be equally reactive with these different strains, possibly leading to cross-strain protection.

As mentioned in the beginning, the influenza virus system served for us as a model. It was not our intention to prepare the most effective commercial vaccine against influenza, but rather to prove the feasibility of the approach and the use of synthetic material for provoking protective immunity towards an animal virus. The influenza virus system offered the advantage that the immune response elicited by the synthetic material can be assessed on four different levels: a) the immunochemical reaction, namely, the capacity of the elicited antibodies to interact with the peptide as such and to cross-react with the intact virus; b) the interference with biological activity of the hemagglutinin molecule, that is to say the capacity of the antibodies to neutralize the hemagglutinin activity of the virus; c) the *in vitro* neutralization of the virus, as expressed by the reduction of the virus plaque formation in tissue cultured cell monolayers resulting from its interaction with the antibodies; and the most crucial criterion: d) the *in vivo* protection of animals as manifested by the decrease in the incidence and the severity of infection following active immunization with the synthetic antigen. The results presented in this manuscript demonstrate that, indeed, the conjugate we have synthesized proved effective on all four levels. It is hoped that the protective effect of synthetic vaccines could be augmented in the future by the use of more adequately designed antigenic determinants, or by the combined use of more than one peptide attached to the carrier molecule.

REFERENCES

Arnon, R. (1972). In: *Immunity of Viral and Rickettsial Diseases*. New York, Plenum Press, p. 209.

Arnon, R. (1974) In: *Peptides, Polypeptides and Proteins*. eds. Lotan, N., Goodman, M. and Blout, E.R. Wiley, N.Y. p.538.

Arnon, R. (1979). *Pharmac. Ther. 6,* 275.

Arnon, R. and Sela, M. (1960). *Biochem. J. 75,* 103.

Arnon, R., Bustin, M., Calef, E., Chaitchik, S., Haimovich, J., Novik, N. and Sela, M. (1976). *Proc. Natl. Acad. Sci. USA 73,* 2123.

Arnon, R., Maron, E., Sela, M. and Anfinsen, C.B. (1971). *Proc. Natl. Acad. Sci. USA 68,* 1450.

Arnon, R., Novik, N., Haimovich, J. and Chaitchik, S. (1977). *Israel J. Med. Sci. 13,* 1022.

Fazekas, De St. Groth, S. and Webster, R.G. (1966). *J. Exp. Med. 124,* 331.

Jackson, D.C., Brown, L.E., White, D.O., Dopheide, T.A.A. and Ward, C.W. (1979). *J. Immunol. 123,* 2610.

Kilbourne, E.D. (1969) In: *Fundamental Techniques in Virology*. eds. Habel, K. and Salzman, N.P. Academic Press, N.Y. p.146.

Langbeheim, H., Arnon, R. and Sela, M. (1976). *Proc. Natl. Acad. Sci. USA 73,* 4636.

Laver, W.G. and Air, G.M., eds. (1979). *Structure and Variation in Influenza Virus*. Elsevier, North Holland.

Laver, W.G., Air, G.M., Dopheide, T.A.A. and Ward, C.W. (1980). *Nature 283,* 454.

Sela, M. (1975). In: *Antiviral Mechanisms. Perspectives in Virology IX*. New York. Academic Press, p. 91.

Teicher, E., Maron, E. and Arnon, R. (1973) *Immunochemistry 10,* 265.

Webster, R.G. and Askonas, B.A. (1980) *Eur. J. Immunol. 5,* 396.

Wiley, D.C., Wilson, I.A. and Skehel, J.J. (1981). *Nature 289,* 373.

Wilson, I.A., Skehel, J.J. and Wiley, D.C. (1981) *Nature 289,* 366.

The latter part of these data was recently submitted for publication in *Proc. Natl. Acad. Sci. USA*.

POEMS

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OWED TO THE PERIPATETIC VIROLOGISTS

Oh, we've roamed through every nation
In pursuit of education
On the structure and the nature of the virus.
We will go to any place
Where there's laboratory space
If the scientist in charge will only hire us.

There's no problem to unravel
That cannot be solved by travel
If the way is paid by NIH or other.
There's no project so bizarre
That we cannot wander far away
From family and fireside and mother.

To the Indies and the Andes
To the desert where it's sandy
We'll pursue the little virus to its lair.
Nothing could be finer than to be in Mainland China
When finally it surfaces for air.

And all of us who wonder
What's so marvelous "down under"
Can visit there to find out what is cooking.
Are the sequences reversed on the
Down side of the earth?
In any case there is no harm in looking!

Over oceans over seases
We pursue the dread diseases
From the Arctic to the sunny tropic clime
And in search of other prey
We vacation on the way
On an island where the moonlight is sublime.

ISBN 0-12-515080-6

On from bosky dells and flowers
On to London and the tower
Assembling in Congresses we chat
So it's really not a pity
To end up in Salt Lake City -
We're learning and that's all there is to that!

A RONDELAY (WITHOUT CADENZA) BY THE VIRION OF INFLUENZA

Now you have me - sucrose-banded,
Enveloped and negative-stranded
Spiked and cleaved and slightly dented
Into pieces eight, segmented -
Without mercy I've been strained
Through filters - then been bromelained, and
Torn apart by each detergent
With a haste unseemly, urgent -
All my helices displayed
Just to show you how I'm made.

My polypeptides have been mapped
My hemagglutinin unwrapped -
All my sequences are clear -
All the way from Arg to Ser.
With techniques sharp and newly honed
My very genes have now been cloned -
Transplanted to an alien host
Wherein my evanescent ghost
As solitary as an elf
Has managed to express myself.

And scientists have labored nights
To probe my antigenic sites
Some fresh from school with new diplomas
(Aided by their hybridomas)
Scramble up trimeric slopes
Counting all my epitopes
And every Ph.D. or pupil
Utterly devoid of scruple
Mates me with complete abandon -
Asks, then, why my genes are random
Can I kiss and never tell
When genotyped upon a gel?
Which, if inspected with acuity
Will document my promiscuity -
Must you write, in fat reports

How flagrantly I reassort?
No boundaries have my misbehavin'
Horsey set or duck or avian -
In other moments less sublime
You've put my perils before swine!

And yet in 1981
Despite the work that has been done
The epidemics come and go
As regular as winter snow.
And people cough and people die
And all of you still wonder why.
I'm so perverse and ever mutable
And so eternally unscrutable.
But think about just what you'd do
If there were really
No more flu!

Edwin D. Kilbourne

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L

M