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An investigation of antigenic drift of neuraminidases of influenza A (H1N1) viruses

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SUMMARY

A newly developed lectin neuraminidase test (LNT) and a panel of mouse monoclonal and post-infection ferret antibodies have been used to analyse antigenic drift in N1 neuraminidases of influenza A viruses isolated between 1933 and 1957 and also between 1977 and 1980. Significant antigenic differences were detected among the 'early' (1933–57) viruses since the NA of viruses isolated one year apart could be distinguished serologically. The NA of the 're-emerged' virus A/USSR/92/77 (H1N1) was antigenically related but not identical to influenza A viruses isolated in 1949 (A/Paris/49 (H1N1), A/Geneva/49 (H1N1)) which thus predates the previously observed antigenic similarity of A/USSR/77 with A/FW/50 (H1N1) virus.

INTRODUCTION

For influenza virus surveillance and for epidemiological studies, investigation of antigenic variation in both neuraminidase (NA) and haemagglutinin (HA) is necessary, but existing methods for analysing antibody levels to NA (Aymard-Henry *et al.* 1973; Schild *et al.* 1974; Callow & Beare, 1976) are relatively insensitive. A novel lectin neuraminidase test (LN-test) based on the specific agglutination of red blood cells after exposure to influenza virus neuraminidase has been developed recently (Luther *et al.* 1982, 1983). Biologically active viral neuraminidases split off *N*-acetyl neuraminic acid from erythrocytes, thus exposing galactose residues in the terminal position of the sugar side chain. These sugar residues react specifically with arachis lectin (peanut lectin) (Bird, 1964). The sensitivity of the LN-test is considerably higher than the conventional neuraminidase test, and antibodies inhibiting neuraminidase can be assayed with a high degree of sensitivity and with relative ease, particularly with large numbers of sera and with post-infection animal (ferret) sera (Luther *et al.* 1982). The present study utilizes the specificity achieved with the LN-test to investigate antigenic relationships of the NA of A/USSR/92/77 (H1N1) virus and of earlier isolated viruses of the same antigenic subtype using mouse monoclonal and post-infection ferret antibodies. We confirm the antigenic relatedness of the NA of A/USSR/92/77 and A/FW/50 viruses described previously (Zhdanov *et al.* 1978; Kendal *et al.* 1978) but establish that the NAs of the two viruses are not

antigenically identical. Furthermore, we find that a similar antigenic relationship with A/USSR/92/77 holds for the NA of viruses isolated the previous year (i.e. 1949) and isolated in different geographical areas of the world.

MATERIALS AND METHODS

Viruses

Influenza A viruses used were from the collection at the National Institute for Biological Standards and Control (London). Recombinants used were NIB-7 (A/Brazil/11/78 × A/PR/8/34 – H1N1), X–53 (A/New Jersey/8/76 × A/PR/8/34 – H1N1), Eq-USSR (A/Equine-1/Prague/56 × A/USSR/92/77 – H7N1). The viruses were grown in embryonated hen's eggs by standard techniques and purified and concentrated by sucrose gradient centrifugation (Skehel & Schild, 1971).

Antibodies to N1 neuraminidase

Monoclonal antibodies (Q/17 and P/2) prepared against the NA of A/USSR/92/77 (H1N1) virus were kindly supplied by Dr R. G. Webster and originally designated 14/3 and 58/1 respectively and were prepared using standard techniques (Webster, Hinshaw & Laver, 1982).

To obtain post-infection polyclonal antisera, ferrets were infected intranasally with virus and bled 12 days later.

Hyperimmune antisera were prepared by immunizing rabbits with two serial injections at 10-day intervals of 50 μg virus protein intramuscularly in Freund's complete adjuvant. A purified recombinant influenza virus was used (A/Equine/Prague/56 × A/USSR/92/77 – H7N1) which possessed the correct NA but an irrelevant HA antigen.

Lectin neuraminidase test (LNT)

The test was carried out as described previously (Luther *et al.* 1983). In brief, a twofold dilution series of the virus suspension was prepared in the test medium (PBS), pH 7·2, in microtitre plates. Twenty μl of a 5% human O erythrocyte suspension was added to 20 μl of each virus dilution followed by an incubation for 18 h at 37 °C. The agglutinability of the erythrocytes was then tested by adding 20 μl arachis lectin. Macroscopical examination for agglutination was carried out 60 min later at room temperature. For detection of neuraminidase-inhibiting antibodies, serum dilution series were prepared in microtitre plates (20 μl). Twenty μl of virus suspension (NA-activity was 4 units, where one unit is equivalent to one titre step-splitting of NANA from erythrocytes) were added to each serum dilution and incubated at room temperature for one hour. Then 20 μl of the 5% human erythrocyte suspension was added and incubated for 18 h at 37 °C. Subsequently 20 μl arachis lectin was added to each serum dilution. Reading was carried out after 60 min incubation at room temperature.

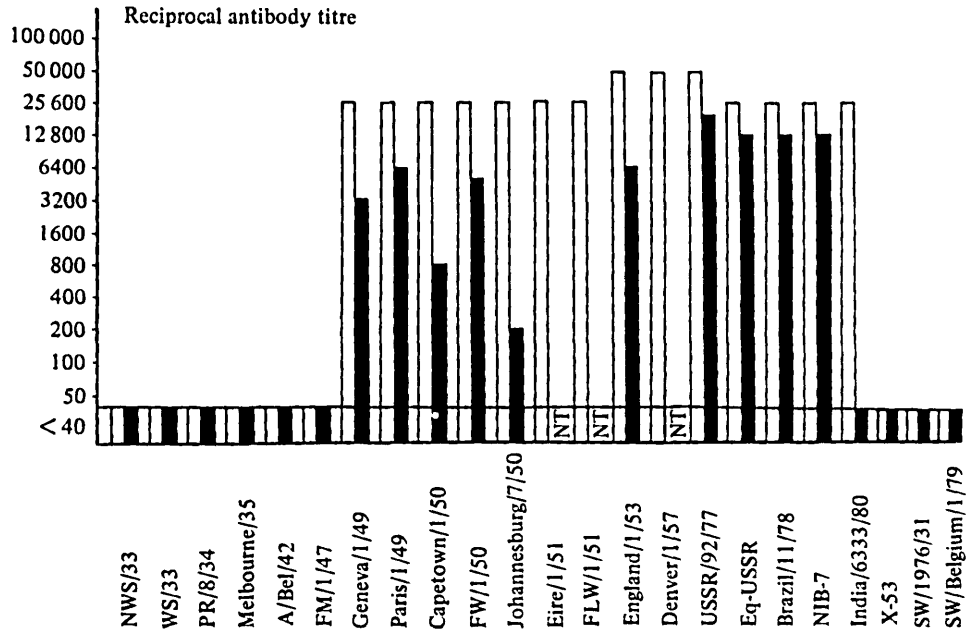

Fig. 1. Antigenic drift of influenza N1 neuraminidase analysed using monoclonal antibodies. □ Q/17, ■ P/2, monoclonal antibodies to A/USSR/92/77 H1N1. NT, not tested.

RESULTS

Serological analysis of influenza A (H1N1) viruses using monoclonal antibodies to NA

Serological reactions between different influenza N1 neuraminidases and two monoclonal antibodies prepared against the NA of A/USSR/92/77 virus are illustrated in Fig. 1. The monoclonal antibodies cross-reacted serologically with the NA of the homologous virus (A/USSR/92/77) and with certain 'early' H1N1 viruses such as A/Geneva/49, A/Paris/49, A/Capetown/50 and A/FW/50. No serological cross-reactivity of the NA of A/USSR/92/77 virus and the NA of earlier isolated viruses such as WS/33, A/PR/8/34 and A/FM/1/47 was detected. The serological specificity of the LN test was controlled by absence of serological reaction with three influenza A/Swine viruses (X-53, A/SW/1976/31 and A/SW/Belgium/79) and, conversely, the cross-reactivity with a recombinant virus A/Eq-USSR which possessed N1 neuraminidase but a serologically different HA. Monoclone P2 was more serologically restricted and failed to cross-react with A/India/6333/80, and also cross-reacted to lower titres with certain early viruses such as A/Jhb/50 and A/Capetown/50.

Previously, a close antigenic relationship between the HA and NA of A/FW/50 (H1N1) and A/USSR/77 (H1N1) viruses has been described (Zhdanov *et al.* 1978; Kendal *et al.* 1978) but the above results suggest, in addition, a significant antigenic relationship between the NA of A/USSR/77 and H1N1 viruses isolated a year earlier (1949) and from different geographical areas.

Table 1. *Serological analysis of NA of 'early' and 'late' influenza A (H1N1) viruses*

Viruses		Monoclonal antibody	Post-infection ferret sera: Anti FM/1/47 F1	Anti Paris/1/49 F32	Anti A/Geneva/49F	Anti FW/1/50 F12	Anti FW/1/50 F18	Anti Johann/7/50 F23	Anti Eire/1/51 F5	Anti FLW/1/51 F10	Anti England/53 F7	Anti USSR/77 F34	Anti Hong Kong/77 F36	Anti Brazil/11/78 F2	Rabbit hyper-immune sera: Rabbit anti-Eq-USSR/77 R 375	Rabbit anti-Eq-USSR/77 R 376
PR/8/34	H1N1	10	10	10	10	8	80	10	10	160	10	20	160	40	5120	1280
FM/1/47	H1N1	10	*1280*	640	160	640	320	320	NT	NT	NT	640	320	640	NT	NT
Geneva/49	H1N1	3200	160	2560	*640*	80	320	640	NT	NT	NT	80	320	2560	12800	12800
Paris/1/49	H1N1	6400	160	*1280*	1280	320	320	2560	NT	NT	NT	320	NT	1280	6400	NT
Capetown/1/50	H1N1	800	NT	320	2560	64	320	640	160	20	1280	320	320	640	NT	NT
FW/1/50	H1N1	6400	40	320	320	*64*	*320*	640	160	10	640	1280	640	640	5120	10000
Johann/7/50	H1N1	200	320	1280	640	64	256	*2560*	320	256	1000	640	1280	128	NT	NT
Eire/1/51	H1N1	256	NT	160	160	320	160	320	*1280*	40	640	80	1280	640	NT	NT
A/FLW/1/51	H1N1	10	160	20	10	16	320	2560	40	*5120*	1600	320	640	320	40000	40000
England/1/53	H1N1	6400	NT	320	40	32	80	2560	128	320	*1600*	320	640	128	40000	40000
Denver/1/57	H1N1	10	10	10	10	10	2	256	80	160	160	20	320	320	640	10000
USSR/0092/77	H1N1	20000	160	2560	2560	128	2560	1280	160	20	640	*2560*	*2560*	2560	20000	40000
Brazil/11/78	H1N1	12800	160	640	320	32	640	320	320	40	320	2560	1280	*5120*	20000	40000
Eq-1-USSR (Equine-Prague × USSR)	H7N1	12800	NT	NT	NT	NT	NT	NT	NT	NT	NT	2560	1280	NT	40000	40000

NT: not tested.
Note. Each result is the mean of duplicate tests.

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Serological analysis of influenza A (H1N1) viruses using post-infection ferret sera

The serological relationships of NAs of viruses within the H1N1 subtype described above were confirmed using post-infection ferret sera (Table 1). Thus, a ferret serum to A/Brazil/78 virus (F2) cross-reacted serologically to a high titre in the lectin test with A/Geneva/1/49, A/Paris/1/49 viruses and to a lesser extent with A/Eire/51 and A/FW/1/50 and A/Capetown/1/50 viruses. A post-infection ferret serum to A/USSR/77 virus (F34) cross-reacted with A/FW/50 and to a lesser extent with A/Paris/49, A/FLW/51 and A/England/53. Conversely, ferret sera prepared against 'early' isolated H1N1 viruses such as A/Geneva/49, A/Paris/49, A/FW/50 and A/Johannesburg/50 cross-reacted serologically with the NA of A/USSR/77 virus to an equivalent titre as to the homologous virus.

Individual ferret sera were obtained with finely differing serological specificity for N1 neuraminidases. Thus F12 and F18, although both prepared against A/FW/50 virus reacted differently with A/USSR/77 virus, the latter serum reacting to higher titre with A/USSR/77 than with the homologous virus, whereas F12 showed maximum reactivity with the homologous A/FW/50 virus. A significant degree of antigenic heterogeneity was detected in the NA of different viruses isolated in the same year (1951), and ferret serum F5 prepared using A/Eire/51 virus showed only low serological cross-reactivity with A/FLW/51 and with viruses isolated in the previous year. Conversely, a ferret serum prepared against A/FLW/51 failed to react serologically with the NA of A/Eire/151 virus.

Hyperimmune antisera to the NA of A/USSR/77 virus reacted to equivalent titres with the homologous virus and the NA of A/FLW/51 and A/England/53 viruses and to a lesser but nevertheless significant extent with A/FW/50, A/Paris/49, A/Geneva/49, A/PR/8/34 and A/Denver/57 viruses. This confirms previous serological studies with NA using hyperimmune sera (Zhdanov *et al.* 1978; Kendal *et al.* 1978) which have a broadly reacting serological specificity.

DISCUSSION

Antigenic relationships within the N1 neuraminidase subtype of influenza A virus have been demonstrated previously by a number of investigators (Panniker, 1968; Kendal *et al.* 1978; Zhdanov *et al.* 1978), but the exclusive use of hyperimmune sera limited the sensitivity of the studies and the possibility of detecting minor antigenic variations in the neuraminidases. The present study is more comprehensive as regards the number of H1N1 viruses analysed whilst, in addition, the newly developed lectin test (Luther *et al.* 1983) enabled us to utilize for the first time the high degree of serological specificity of post-infection ferret sera which has been well established in studies with influenza HA (reviewed by Schild & Dowdle, 1975).

In particular, previous serological studies have described an antigenic relationship between the NA of the 'early' virus A/FW/50 and the 're-emerged virus' A/USSR/77 (Kendal *et al.* 1978; Zhdanov *et al.* 1978). This serological relationship is confirmed in the present study but is extended to include other influenza A (H1N1) viruses isolated the previous year (1949) in Europe rather than the USA. Serological analysis of the NA of such viruses was carried out using monoclonal

antibodies and post-infection ferret sera. Both monoclonal antibodies against the NA of A/USSR/77 virus reacted to equivalent NI titres with the homologous virus and the early viruses A/Geneva/49, A/Paris/49 and A/FW/50. Also, a post-infection ferret serum prepared with A/USSR/77 virus (F34) cross-reacted with the NA of A/England/53, A/FLW/51, A/FW/50 and A/Paris/49 viruses, whilst serum from a ferret infected with A/FW/50 virus also reacted serologically to significant extent with the NA of A/Paris/49, A/Eire/51 and A/USSR/77 viruses. This would indicate, therefore, that a possible progenitor virus of A/USSR/77 was A/Paris/49 or a virus circulating at least a year before A/FW/50. Existing biochemical data, which indicate a close genetic relationship between A/USSR/77 and A/FW/50 viruses by RNA:RNA hybridization (Scholtissek, von Hoyningen & Rott, 1978), oligonucleotide mapping (Nakajima, Desselberger & Palese, 1978) and nucleotide sequencing (Block & Air, 1982) would not be in conflict with the present serological data since both RNA:RNA hybridization at 87 °C (Scholtissek *et al.* 1978) and analysis of oligonucleotide spots of the total and gene segment RNA of A/FW/50 and A/USSR/77 (Kozlov *et al.* 1981) showed some genetic differences between the NAs of the two viruses. Block & Air (1982) have examined sequence variation at the 3′ end of the neuraminidase of certain early and late H1N1 viruses, and although the data indicate a 96% nucleotide homology between the corresponding sequences of the NA of A/FW/50 and A/USSR/77 viruses, no virus isolated in 1949 such as A/Paris/49 described here was examined. In addition, the region sequenced is not represented in soluble peptides isolated from NA heads which would contain the antigenic areas, but rather the tail of the neuraminidase which is not known to be antigenically active, and hence the sequence variation may be considerably more in the antigenic area. Finally, our own electrophoretic analysis of ss RNAs of A/FW/50 and A/USSR/77 using a sensitive technique for showing genetic differences between closely related viruses (reviewed by Palese & Young, 1982; Hugentobler, Schild & Oxford, 1981; Palese & Schulman, 1976) showed a significant electrophoretic migration difference in gene 6 (NA) between the two viruses (J. S. Oxford, unpublished data).

Data from utilization of both the lectin assay and monoclonal antibodies or post-infection ferret sera would suggest that the antigenic structure of the NA within the N1 subtype is more complex than previously observed using hyperimmune sera (Kendal *et al.* 1978). Thus, using hyperimmune sera to NA, Paniker (1968) concluded that members of the A0–A1 subtype isolated from 1934 to 1956 were all interrelated, with no clear-cut antigenic difference between A0 and A1. Our results indicate a much more complex series of antigenic relationship. Thus, post-infection ferret sera reacted serologically with the NA of the homologous viruses A/Eire/51 and A/FLW/51, but poorly with viruses isolated the same or only the previous and following years. We recognize that our serological analysis could be extended using more H1N1 viruses and additional post-infection ferret sera. Webster, Hinshaw & Laver (1982) have used monoclonal antibodies to suppress the replication of parental virus and to allow the emergence of antigenic mutants, and these experiments indicate the presence of three or four non-overlapping antigenic areas on the NA antigen. A large number of monoclonal antibodies will be required, therefore, for a comprehensive analysis of these H1N1 viruses. Finally, the lectin test under certain conditions has the technical problem

of the previous NI test (Aymard-Henry *et al.* 1973), namely steric hindrance, although this parameter was controlled to a certain extent in the present study by the use of recombinant influenza viruses.

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