The haemagglutinins of influenza A (H1N1) viruses in the ‘O’ or ‘D’ phases exhibit biological and antigenic differences

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SUMMARY

Influenza A (H1N1) viruses when initially isolated in mammalian cell cultures (MDCK cells) had different agglutination reactions with chicken and guinea-pig erythrocytes compared to the same viruses after passage. On first isolation the virus HA resembled the ‘O’ phase viruses described originally by Burnet and Bull and agglutinated mammalian but not avian erythrocytes. After passage, the virus HA resembled a classical ‘D’ phase virus and agglutinated both avian and mammalian erythrocytes. Monoclonal and polyclonal antisera detected antigenic differences between the HAs of the viruses in the ‘O’ and ‘D’ phases. The ‘O’ phase virus HA reacted preferentially with antibodies in post infection human antisera. Viruses in the ‘O’ phase replicated poorly in the allantoic cavity of embryonated hens’ eggs whilst ‘D’ phase virus replicated in both MDCK cells and in embryonated hens’ eggs. At least three distinguishable subpopulations of influenza A (H1N1) viruses may co-exist in clinical throat swab material, including viruses possessing HAs in the ‘O’ and ‘D’ phases and other ‘D’ phase viruses cultivable in embryonated hens’ eggs but antigenically distinguishable from the corresponding ‘D’ phase virus in MDCK cells.

INTRODUCTION

Burnet and Bull [1] first reported that some strains of influenza A (H1N1) viruses exist in two biologically distinguishable forms which were termed ‘O’ (original) and ‘D’ (derived). These two biological phenotypes described by Burnet and Bull [1] were distinguishable on the basis of erythrocyte agglutination properties: the ‘O’ form of the virus haemagglutinin (HA) agglutinated guinea-pig erythrocytes but not chicken erythrocytes whilst the ‘D’ form derived from the ‘O’ phase by passage in the allantoic cavity of embryonated hens’ eggs, agglutinated erythrocytes of both species. The ‘O’ form grew only in the amniotic cavity of the chick embryo, whereas the passaged ‘D’ form could be cultivated in

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either the allantoic or amniotic cavity suggesting that the two HAs possessed different receptor affinities (reviewed in [2]). Clinical samples were presumed to contain both phenotypes of the virus. No antigenic differences were detected between 'O' and 'D' phase HAs using post infection ferret antisera.

More recent studies of the cultivation of influenza A (H1N1) viruses in eggs or mammalian cells have also implied that clinical samples contain mixtures of at least two distinguishable HA phenotypes [3-9]. Thus, influenza A and B viruses isolated from clinical samples and cultivated in mammalian cells, such as MDCK cells, possess HAs which are antigenically and biochemically distinct from viruses from the same clinical sample but isolated in embryonated hens' eggs. It is presumed that these differences arise as a consequence of the different selective pressure on the receptor binding site of the virus HA [10-14]. The molecular basis of this phenotypic variation is either single and double amino-acid substitutions at, or in close proximity to the receptor binding site on the HA and near to epitope B [15].

The present study describes for the first time antigenic and biochemical differences between HAs of influenza viruses in the 'O' and 'D' phenotypes and also investigates the possible relationship of 'O' and 'D' forms of virus to Madin-Darby canine kidney (MDCK) cell and egg grown influenza A (H1N1) viruses. We conclude that 'O' to 'D' variation is different from MDCK or egg induced variation and presents an additional source of genetic and phenotypic variation of influenza A viruses.

MATERIALS AND METHODS

Virus samples

The throat gargles from persons ill with influenza were inoculated directly into either MDCK cell cultures grown in Eagles minimal essential medium containing 10 µg/ml trypsin (Difco) or into the amniotic cavity of embryonated hens' eggs by standard techniques [2]. After isolation the viruses were then passaged further as undiluted fluids in MDCK cells or in the allantoic cavity of embryonated hens' eggs respectively.

Monoclonal antibodies

Monoclonal antibodies (MAbs) to the HA of influenza A/Brazil/11/78, A/England/333/80, A/Baylor/5700/82, A/Baylor/1515/82 and A/Christ's Hospital/91/83 (H1N1) viruses were prepared using standard procedures [16]. A small panel of MAbs was also prepared using virus in the 'O' and 'D' phase. Mice were immunized twice with purified influenza A (H1N1) egg grown viruses ('D' phase), or MDCK cell grown virus ('O' phase). Spleen cells were removed for fusion 4 days after the second dose of antigen, which was given intravenously. Antibody for serological analyses was prepared as mouse ascitic fluid.

Serological tests

For ELISA assays, sucrose gradient purified, concentrated virus, diluted to 256 HA units (HAU) per 50 µl was used as antigen. Non-specific sites were blocked with 3% Marvel and bound antibody detected with biotinylated anti-mouse Ig

whole antibody, using the streptavidin-horseradish peroxidase system (Amersham International).

Haemagglutination inhibition (HAI) tests were performed in microtitre plates by standard methods (reviewed in [2]) employing 0.5% v/v guinea-pig erythrocytes except where indicated in the tables.

Plaque reduction neutralization (PRN) tests were also carried out with 50 p.f.u. of virus incubated at room temperature with doubling dilutions of sera for 1 h [17]. The mixtures were titrated for residual infectivity in MDCK cells with agar overlay. Plaques were counted after 4 days of incubation at 30 °C and the serum titre expressed as the dilution which gave a 50% plaque reduction.

Human polyclonal sera

Sera were obtained from children (aged < 10 years old) or adults (aged 21–30 and > 51 years old) in Florence, Italy as part of a serological survey in 1984. The sera were treated with 0.5% KIO₄ for 20 min to destroy non-specific inhibitors followed by neutralization of the KIO₄ with 10% glucose for 30 min at room temperature. Additional sera were also tested from young adults and pretreated with receptor destroying enzyme (RDE Philips Duphar, Weesp, Holland). In previous surveys we have assumed that detectable HAI antibody in such sera result from natural virus infections in the community.

RESULTS

The influenza A (H1N1) viruses isolated and subsequently passaged in mammalian (MDCK) cells were analysed to determine whether such isolates possessed 'O' or 'D' phase phenotypes because these had only been described previously in viruses grown in embryonated hens' eggs. Examination of the agglutination patterns of the viruses with chicken and guinea-pig erythrocytes established that all direct virus isolates on first growth in MDCK cells were in the 'O' phase (Table 1). Serial passage of viruses in MDCK cells resulted in a change of biological phenotype from the 'O' to the 'D' phase, although a few strains such as A/Florence/1/84 remained in the 'O' phase (Table 1).

Antigenic differences of HA of viruses in the 'O' and 'D' phases

The serological reactivity of HAs in the 'O' and 'D' forms with monoclonal antibodies is shown in Table 2. There was a clear antigenic difference between the HAs in the two phases. Some MAbs against the MDCK-grown virus A/Christ's Hospital/91/83 were identified (e.g. MAbs 339 and 384) which reacted with the 'O' form of the virus and did not react with the 'D' phase of the virus. MAb 41 was shown previously to react exclusively with influenza A (H1N1) viruses grown in MDCK cells [4], and reacted only with 'O' phase virus HA. However the other monoclonal antibodies tested (491, 485 and 336) reacted with both 'O' and 'D' phase HAs. Therefore the antigenic reactivity of the HA of 'O' phase virus and viruses cultivated in MDCK cells and possessing a 'cell-like' antigenic phenotype was different [3].

Table 1. *Erythrocyte agglutination patterns of influenza A (H1N1) virus isolates*

Virus and passage history	HA titre versus		Phase
	Chick erythrocytes	Guinea-pig erythrocytes	
A/F/13/83			
MDCK 1*	< 1	16	'O'
MDCK 8†	16	16	'D'
A/F/19/83			
MDCK 1	< 1	32	'O'
MDCK 8	32	32	'D'
A/F/2/84			
MDCK 1	< 1	32	'O'
MDCK 9	16	16	'D'
A/F/1/84			
MDCK 1	< 1	32	'O'
MDCK 15	< 1	32	'O'

* Numbers denote passages in MDCK cells.

† Change from O to D was detected at this passage level.

The A/F/13/83 virus isolated in MDCK cells in the 'D' phase was also grown in embryonated hens' eggs and maintained a 'D' phase phenotype. However, analysis of these two 'D' phase virus preparations with MAb detected serological differences as exhibited with MAb 26 which only reacted with the 'D' virus phase cultivated in MDCK cells, and not with the 'D' phase HA of virus cultivated in embryonated hens' eggs (Table 2).

We wished to determine if representative MAb which either identified 'O' phase virus (MAb 384) or failed to differentiate 'O' and 'D' phase HAs would also react in virus neutralization tests. MAb 384 and 491 were tested in a plaque neutralization test with 'O' and 'D' forms of A/Florence/13/83 and A/Florence/2/84. MAb 491 neutralized both phenotypic forms at a titre of 6250 whilst MAb 384 neutralized only the 'O' form, thus confirming the HAI results.

Our previous studies with influenza A (H1N1) viruses cultivated in MDCK cells and eggs showed that the former virus preferentially reacted with human sera [4]. The antigenic reactivity of A/Florence/2/84 in the 'O' and 'D' phases with polyclonal human sera is shown in Table 3. The 'O' form of the antigen reacted to a significantly higher titre with sera (35/178 sera with an HAI titre > 40) when compared to the 'D' phenotype (7/178 sera with an HAI titre > 40). Similar results were obtained with A/Florence/13/83 virus.

Infectivity of 'O' or 'D' phase viruses for MDCK cells and eggs

To investigate further any biological relationships of the 'O' and 'D' forms to mammalian (MDCK) and egg grown viruses, we titrated the infectivity of 'O' and 'D' forms of A/Florence/2/84 and A/Florence/13/83 for mammalian cells and eggs (Table 4). The 'O' form was less infective in the allantoic cavity compared to MDCK cells, whereas the 'D' form was equally infectious in both substrates. Thus the 'O' phase virus is similar to MDCK grown virus in its restricted growth in eggs while the 'D' phase virus is comparable to egg grown virus in this respect.

Table 2. *HAI reaction of 'O' and 'D' forms of influenza A (H1N1) viruses with monoclonal antibodies*

Virus Phase	Monoclonal antibodies to A/Christ/91/83 (MDCK)										Monoclonal antibodies to egg grown*			
	336	339	375	377	379	384	485	491			A/Brazil /11/78	A/Baylor /5700/82	A/Baylor /5700/82	A/Baylor /1515/82
A/F/13/83														
O	6400	3200	<†	<	<	1600	> 12800	> 12800			3200	1600	NT†	3200
D (egg)§	NT	NT	NT	NT	NT	<	> 12800	< NT			NT	NT	<	9600
D	6400	<	<	<	<	<	> 12800	> 12800			6400	<	4800	6400
A/F/19/83														
O	6400	6400	6400	<	<	1600	> 12800	> 12800			3200	1600	NT	3200
D	6400	<	<	<	<	<	> 12800	> 12800			6400	<	NT	3200
A/F/2/84														
O	6400	6400	6400	6400	1600	1600	> 12800	> 12800			<	3200	NT	3200
D	1600	<	1600	400	200	<	> 12800	> 12800			<	<	NT	<

* Note that in previous studies MAb 41 reacted preferentially with MDCK cell grown virus, whilst MAb 29 reacted preferentially with egg grown virus. MAbs 17 and 26 were cross-reactive with both MDCK cell and egg grown viruses, whilst MAb 26 was not reactive with use isolates from 1983 (J.S. Oxford, unpublished).

† <, less than 1/100.

‡ NT, not tested.

§ Virus passaged in eggs and phenotypically 'D' phase.

|| Virus passaged in MDCK cells and phenotypically 'D' phase.

Table 3. *Serological reactivity of human antisera to influenza A/Florence/2/84 virus in the 'O' or 'D' phase*

Phenotypic form of virus	Passage history of virus	Number (%) of sera with HAI titre to A/Florence/2/84					
		< 20		> 20		> 40	
O	MDCK 1	114	(64.0)	64	(35.9)	35	(19.7)
D	MDCK 10	154	(86.5)	24	(13.5)	7	(3.9)

Table 4. *Infectivity of 'O' and 'D' viruses in MDCK cultures and eggs*

Virus Phase	Passage history of virus	Infectivity (ID ₅₀) MDCK cells	Egg allantoic cavity
A/F/13/83			
O	MDCK 1	6.0	3.1
D	MDCK 8	6.7	5.7
A/F/2/84			
O	MDCK 1	6.4	< 1.5
D	MDCK 8	7.2	6.3

DISCUSSION

Haemagglutination inhibition and virus neutralization tests with MABs produced against the MDCK cell-grown virus A/Christ/91/83 (H1N1) demonstrated that 'O' and 'D' forms of influenza A (H1N1) viruses were antigenically distinguishable. Certain MABs reacted only with the 'O' form of these viruses and did not react, or reacted poorly, with the 'D' form and this is particularly evident with the A/Florence/2/84 virus. Polyclonal human sera were more likely to react with A/Florence/2/84 HA in the 'O' phase and to higher titre. This result may suggest that the 'O' form of the virus HA is antigenically more related to viruses in clinical isolates than is the HA of the 'D' phase of the virus. Similar data has been described with virus grown in MDCK cells [4] and human sera react to higher titre with the HAs of MDCK cell grown viruses compared to the HAs of egg-grown virus.

Mab 41 which reacted with MDCK grown viruses and not egg grown viruses in previous studies [4] showed serological reactivity with the 'O' phase virus, also suggesting antigenic relationship, but not identity.

Yates and colleagues [19] have mapped two sites recognized by certain of the monoclonal antibodies used in our study to sites Sa and Sb [20] of the H1 haemagglutinin. Thus monoclonal antibody 339, which reacted with 'O' phase virus but not 'D' phase virus HA is known to recognize antigenic site Sa. In contrast, monoclonal antibody 336, which does not distinguish between 'O' and 'D' phases (Table 2) recognizes antigenic site Sb (residue 189). The site recognized by monoclonal antibody 384 could not be mapped because since escape mutants derived from it mapped in different positions on the HA. This antibody may recognize some structural feature of the HA such as carbohydrate and not a specific epitope. Monoclonal antibody 485 is unusual in that variants require two substitutions to escape neutralizing activity [19].

None of the MABs tested in the present study were able to react exclusively with

the 'D' form of the virus without also reacting with the 'O' phase of the same virus. Burnet and Bull [1] were not able to distinguish antigenically between 'O' and 'D' phenotypes of influenza A (H1N1) viruses. However we have recently prepared MABs which react exclusively with the homologous 'O' phase virus both by ELISA and HI tests (JSO unpublished data).

Furthermore our studies show clearly that the 'O' and 'D' phenotypic variation described by Burnet and Bull [1] is not restricted to viruses isolated and cultivated in embryonated hens' eggs as in their original studies: we describe the same findings in viruses isolated and passaged in MDCK cells. The relative genetic instability of the 'O' phase and the selection of 'D' phase virus after passage is also noted here with viruses cultivated and passaged in MDCK cells. As noted originally by Burnet and Bull [1] occasional viruses are maintained in the 'O' phase after passage in embryonated hens' eggs and in our study the virus A/F/1/84 was passaged 15 times in MDCK cells without detectable change from the 'O' to the 'D' phase.

In preliminary experiments and using standard techniques [7, 8, 18] we observed biochemical differences between the HA of A/Florence/2/84 in the two phases (J. Robertson and J. S. Oxford, unpublished). Of particular significance may be glycosylation site differences between the HAs. The 'D' phase of the A/Florence/2/84 virus analysed possessed a potential glycosylation site at residue 144. We have noted before [21] that changes in influenza B virus in this region of the HA may exert profound biological effects on the virus. In the latter study, changes at amino acid position 141, co-varied with attenuation. This region borders the receptor binding site and would be expected to exert effects related to receptor binding such as binding to erythrocytes of different species, the predominant biological characteristic of 'O' and 'D' phase viruses. Sequence analysis of additional pairs of viruses in the 'O' and 'D' phases is required to corroborate these findings.

Our present results suggest that certain influenza A (H1N1) viruses may be present in clinical samples in at least three subpopulations. The 'O' and the 'D' subpopulations are clearly distinguishable by biological, biochemical and antigenic characters. A third virus, biologically similar to the MDCK-grown 'D' form, but antigenically different (Table 2, MAb 26), can be isolated from a clinical sample and cultivated in the allantoic cavity of chicken embryos.

The 'O' phase virus, originally described by Burnet and Bull [1] as growing only in the amniotic cavity of fertile hens' eggs but, as described here, cultivable in MDCK cells, may be the most predominant virus in clinical specimens. Therefore it could represent the choice as a reagent in the HI test for serological studies to assess humoral immunity against influenza A (H1N1) virus in humans. It is biologically indistinguishable by the absence of haemagglutinating properties with chick erythrocytes compared to influenza A (H1N1) viruses 'conventionally' isolated in MDCK cells. Moreover the 'O' phase virus is not genetically stable because limited passage selects out a 'D' phase HA. It can also be hypothesized that the use of viral populations with antigenic and biological characters similar to the 'O' form could stimulate a more protective immune response than the current egg-grown virus vaccine and such studies of vaccine reformulation are at present in progress. Finally, we have isolated an influenza A (H3N2) virus which exhibits a comparable 'O' and 'D' phase variation (A. Azzi, unpublished)

indicating that this form of phenotypic and genetic variation may be more universal than previously considered and not restricted to Influenza A (H1N1) viruses.

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