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Use of *ex vivo* and *in vitro* cultures of the human respiratory tract to study the tropism and host responses of highly pathogenic avian influenza A (H5N1) and other influenza viruses

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ABSTRACT

The tropism of influenza viruses for the human respiratory tract is a key determinant of host-range, and consequently, of pathogenesis and transmission. Insights can be obtained from clinical and autopsy studies of human disease and relevant animal models. Ex vivo cultures of the human respiratory tract and in vitro cultures of primary human cells can provide complementary information provided they are physiologically comparable in relevant characteristics to human tissues in vivo, e.g. virus receptor distribution, state of differentiation. We review different experimental models for their physiological relevance and summarize available data using these cultures in relation to highly pathogenic avian influenza H5N1, in comparison where relevant, with other influenza viruses. Transformed continuous cell-lines often differ in important ways to the corresponding tissues in vivo.

The state of differentiation of primary human cells (respiratory epithelium, macrophages) can markedly affect virus tropism and host responses. Ex vivo cultures of human respiratory tissues provide a close resemblance to tissues in vivo and may be used to risk assess animal viruses for pandemic threat. Physiological factors (age, inflammation) can markedly affect virus receptor expression and virus tropism.

Taken together with data from clinical studies on infected humans and relevant animal models, data from ex vivo and in vitro cultures of human tissues and cells can provide insights into virus transmission and pathogenesis and may provide understanding that leads to novel therapeutic interventions.

1. Introduction

The tropism of influenza viruses for the human respiratory tract is a key determinant of host-range, and consequently, of pathogenesis and transmission. Much of the available data on the tropism of influenza viruses in the human respiratory tract has been derived from autopsy studies of fatal influenza. While this provides important insights into the cells and tissues infected by different respiratory viruses, these data are of necessity restricted to late-stages of severe influenza disease, often following mechanical ventilation. Of the available animals models, only ferrets (and possibly guinea pigs in relation to transmission) are regarded as relevant surrogates for human influenza infection. Nevertheless, ferrets are not humans, and studies of the human respiratory tract remains of paramount importance.

The human respiratory tract is sub-divided into the upper respiratory tract including the nasopharynx and larynx, the conducting airways (trachea and bronchi) and the lower respiratory tract which comprises of the lung parenchyma. Each of these anatomical regions has different types of epithelial cells and influenza viruses differ in their tropism for different cell types at these sites.

The tropism of an influenza virus for the upper, conducting and lower airways has consequences for transmission and pathogenesis. Viruses with tropism for the alveolar epithelium may well cause severe viral pneumonia but may be less transmissible from person-to-person, while those that infect the upper airways are likely to be more transmissible but may cause less severe disease. Thus, experimental models that reflect these anatomical and physiological differences are relevant.

Available experimental models used for assessment of virus tropism and replication competence in humans include (a) *ex vivo* cultures of human respiratory tissues removed at biopsy or surgical excision which are excess to diagnostic requirements, (b) primary

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cells from the human respiratory tract, used with or without differentiation in air–liquid interface cultures, or (c) continuous cell lines derived from the human respiratory tract (*e.g.* A549) or cell lines derived from other animal tissues (*e.g.* Mardin–Darby Canine Kidney (MDCK)).

Experimental use of *ex vivo* cultures of human respiratory tissues removed at biopsy or surgical excision was first adopted in 1960s (Higgins et al., 1969; Roome et al., 1971; Tyrrell and Blamire, 1967; Tyrrell and Hoorn, 1965) as attempts to provide substrates for the isolation and growth of respiratory viruses. Subsequently, for convenience and reproducibility, semi-continuous and transformed continuous cells from various species have been used for the purposes of diagnostic virology. Primary and semi-continuous cell cultures were derived from aborted human embryos (*e.g.* human embryo lung fibroblast cultures) and from non-human primates (*e.g.* primary and secondary Rhesus monkey kidney epithelial cell cultures). Continuous transformed cell cultures used for influenza virus studies include MDCK cells and A549 cells derived from a type II pneumocyte lung carcinoma (Giard et al., 1973). These continuous cell lines require the addition of exogenous trypsin for cleavage of the precursor hemagglutinin molecule (HA_0) to the HA1 and HA2 components of the mature virion.

While these different cell substrates may be useful for virus isolation, diagnostics and vaccine production, they are not equally useful for providing insights on the tropism of influenza viruses in the human respiratory tract. In this review, we summarize available data on influenza virus receptor distribution, the profiles of virus binding and on virus infection and replication in relevant experimental models and compare these with what is known for the human respiratory tract, to identify physiologically relevant experimental systems for investigating the tropism of influenza viruses for the human respiratory tract.

2. Distribution of influenza virus receptors

Sialic acids linked to proteins or lipids on the cell surface are binding receptors for influenza viruses. Studies in the 1980s using desialylated and resialylated red blood cells demonstrated that human influenza viruses preferentially bind α2–6 Gal-linked sialic acids while avian influenza viruses preferentially attach to sialic acids with an α2–3 linkage (Rogers and Paulson, 1983). We used the lectin *Sambucus nigra* agglutinin (SNA) which binds α2–6Gal, *Maackia amurensis* agglutinin (MAA) I which has a preference for α2–3Galβ1–4GlcNAc and MAAII which has a preference to bind α2–3Galβ1–3GalNAc to compare the glycan expression in *ex vivo* cultures of the human nasopharynx, bronchus and lung alveolar tissues with corresponding tissues from patients at autopsy who died of non-respiratory causes (Table 1). These findings are also compared with the glycan expression profiles in undifferentiated and differentiated primary human cell cultures (Chan et al., 2010b; Yu et al., 2011).

Table 1
Summary table comparing the expression of sialic acid receptor of *ex vivo* organ culture models, *in vitro* primary epithelial cell cultures and continuous cell lines of human respiratory tract to the nasopharynx, bronchus and lung of humans, using lectin binding method.

Anatomical site	Culture Model	Differentiation State	Lectin		
			MAAI	MAAII	SNA
			α2-3 N-linked	α2-3 O-linked	α2-6
Nasopharynx	In vivo		++	-	++
	Ex vivo		++	-	++
	In vitro	Undifferentiated	++	+	-
Bronchus	In vivo		++	-	++
	Ex vivo		++	-	++
	In vitro	Undifferentiated	++	++	+
		Well-differentiated	++	-	++
	Cell line	Calu-3 polarized	O-linked α2-3 ≥ α2-6 (Zeng et al., 2007)		
Lung	In vivo		+	++	+
	Ex vivo		+	++	+
	In vitro	Type-I like	++	++	+
		Type-II like	++	+	+
	Cell line	A549 (Type II-like), NCI-H661 cells	α2-3 << α2-6 (Guo et al., 2009; Sieben et al., 2012)		

Overall, there is good concordance between the expression of the sialic acid receptors in the *in vivo* tissues and *ex vivo* organ cultures as well as the well-differentiated primary cell culture models but the receptor distribution in some undifferentiated primary human cell cultures and continuous cell lines do not correspond well with what is found in corresponding human tissues (Table 1).

In humans, an almost complete differentiation of nasopharyngeal epithelium is found in a 12–13 week fetus *in utero* with two distinct cellular types, ciliated and microvillus-provided cells (Gulisano et al., 1992). The nasopharyngeal epithelium has five characteristic cellular types found on the surface, including the tall cylindrical ciliated cells; cylindrical cells covered only with microvilli, secretory cells, flat epithelial cells, cubodial cells with microvilli on the surface and many vesicles in the cytoplasm (Karchev and Kabakchiev, 1984). The cell surface of the nasopharyngeal epithelial cells is decorated with glycans with different terminal residues including sialic acids (Chew et al., 1991). Lectin binding studies of *ex vivo* nasopharyngeal, traceho-bronchial and lung organ cultures accurately reflect the receptor profile observed in normal human tissues (Nicholls et al., 2007a,b).

In endemic areas, exposure of humans to H5N1 infected poultry is very common but H5N1 disease remains exceedingly rare. It has been hypothesized those who get H5N1 disease may have differences in the distribution of avian influenza binding Sia α2–3 receptors in the respiratory tract. In a clinical study, the differences of sialic acid receptors distributed in different human tissues, including extrapulmonary organs, in H5N1-infected and non-infected patients was compared using lectin histochemistry (Yao et al., 2008). This study used biotinylated MAAII and SNA

Table 2
Attachment of labeled influenza virions to human respiratory epithelia for comparison with distribution of influenza receptors by lectin binding (see Table 1).

	HPAV H5N1	Seasonal H1N1	Seasonal H3N2	H7N7 (2003)	H1N1pdm (2009)
Virus attachment					
Conjunctiva	No data	No data	No data	No data	No data
Nasal/Nasopharynx	No (scant binding to nasopharynx and paranasal sinuses, ciliated cells)	Yes (ciliated > goblet cells)	Yes (ciliated > goblet cells)	No Data	Yes (Ciliated > goblet cells)
Trachea	No	Yes	Yes	No data	Yes
Bronchus	Minimal	Yes	Yes	No data	No data
Bronchiole	Yes	No	Minimal	Yes	No data
Lung	Yes (Type II)	Yes	Yes	Yes	No data
References	Chutinimitkul et al. (2010), van Riel et al. (2006, 2007)	van Riel et al. (2010, 2007)	Chutinimitkul et al. (2010), van Riel et al. (2007)	de Wit et al. (2010)	van Riel et al. (2010)