The Localization of Influenza Virus
in the Respiratory Tract of Ferrets: the Different Susceptibilities of Fresh and Maintained Organ Cultures

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SUMMARY

Tissues of the upper respiratory tract of ferrets are more susceptible to influenza virus infection than those of the lower respiratory tract. An organ culture technique has been standardized sufficiently to examine the basis for these differences.

In experiments with an Asian and two Hong Kong influenza viruses maintenance of organ cultures for 24 h before inoculation consistently and markedly increased the susceptibility of lung, the least susceptible tissue. The susceptibility of the most susceptible tissue, nasal turbinates, was increased significantly (but to a lesser extent than lung) in some experiments but not others. Tracheal organ cultures showed two patterns of susceptibility; about half were poorly susceptible but showed a marked increase with maintenance. The remainder were moderately susceptible and showed no increase with maintenance.

The increased susceptibility of lung with maintenance was not due to fibroblast outgrowth, to loss of inhibitory material easily removed by washing, to killing of commensal bacteria by the bactericidal medium nor to changes in mucus distribution. Although central necrosis occurred in the maintained tissue pieces it was not correlated with increased susceptibility and virus replicated only in peripheral cells which remained healthy throughout culturing. The increase in susceptibility was demonstrated after maintenance for 6 h and increased to a maximum by 24 h.

The reason for the low susceptibility of the lung in vivo may be established by comparing organ cultures of fresh lung and nasal turbinates and by analysing the basis for the increased susceptibility of the lung with maintenance.

INTRODUCTION

Following intranasal inoculation of ferrets with an Asian strain of influenza virus [A/Moscow/1019/65 (H2N2)] virus yields were a 100-fold higher in the nasal turbinates than in the lung and trachea and no significant quantities of virus were detected in 16 other tissues (Basarab & Smith, 1969). In organ culture the patterns of virus replication in respiratory tissues paralleled those in the tissues in vivo; virus replicated more rapidly and to higher titres in the nasal turbinates than in the lung and trachea (Basarab & Smith, 1970; Toms, Rosztoczy & Smith, 1974). The reasons for the differences in susceptibility might then be investigated by comparative studies in organ culture. As a beginning, Toms et al. (1974) showed that the kinetics of replication over one infectious cycle were similar for nasal turbi-

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nates, trachea and lung, new virus appearing from all tissues at about the same time (5 to 6 h) after inoculation. This suggested that the susceptibility differences may be determined either at the initiation of infection or by the amount or quality (as regards infecting other cells in the organ culture) of the virus released (Toms et al. 1974).

In continuing the use of organ cultures several technical factors were considered. Firstly, a reproducible system amenable to statistical analysis was required. Secondly, organ cultures can show functional and histological differences from tissues in vivo (Herbstlaier, 1970; Rubenstein & Tyrrell, 1970; Pavelka, 1975). In previous studies of tissue specificity of influenza virus (Basarab & Smith, 1970; Toms et al. 1974) as in other systems (Hoorn & Tyrrell, 1969), organ cultures were maintained for 1 to 2 days before inoculation and changes affecting susceptibility might have occurred during this time.

This paper describes standardization of the organ culture technique and the finding that maintenance of lower respiratory tract tissues—particularly lung—results in a marked increase in susceptibility compared with that of fresh tissues.

**METHODS**

**Strains of influenza virus.** Strain A/Moscow/1019/65 (H2N2) (abbreviation; A2M) and the preparation of seed stocks were described by Basarab & Smith (1969). Working stocks (prepared every 6 months) were prepared by intra-allantoic inoculation of seed stock [10² 50% egg infectious doses (EID₅₀)] into 10-day-old embryonated hen eggs which were harvested 20 h post-inoculation. Mean infectivity titres were 7·2 log₁₀ 50% egg bit infectious doses (EBID₅₀); 8·5 log₁₀ EID₅₀/ml and HA titres were 1/8 to 1/32. Clones 7a and 64d of the recombinant virus A/PR/8/34—A/England/939/69 (H₃N₂) were described by Toms et al. (1976) together with the preparation of seed and working stocks.

**Ferrets.** These (6 to 24 months old; males, 1 to 1.5 kg; females, 0.6 to 1.0 kg) were obtained from A. S. Roe, Norfolk. Serum HI titrations showed them to be non-immune.

**Infectivity (EBID₅₀, EID₅₀), haemagglutination (HA) and haemagglutination inhibition (HI) assays.** These were as described by Toms et al. (1974).

**Organ cultures.** These were prepared by a modification of the method of Basarab & Smith (1970) described by Toms et al. (1976). Six pieces of tissue, taken randomly from a large number cut from the organ of one animal, were placed in each culture dish and at least four dishes were used for each tissue or tissue treatment. Cultures prepared as quickly as possible after removal of tissue from the animals were inoculated either after 1 h at 37 °C (fresh cultures) or after 24 h at 37 °C (maintained cultures). In both cases the medium (1·5 ml per dish) was changed before inoculation. The pieces were inoculated with 4·6 log₁₀ EBID₅₀ virus per dish unless stated otherwise as described by Toms et al. (1976). Supernatant fluid from the dishes was harvested every 24 h and stored at −70 °C for assay. In comparisons between different tissues and treatments, samples taken from the same time point were formally randomized (Fisher & Yates, 1963) and titrated at the same time to minimize differences due to error of assay. Differences were analysed by one way analysis of variance and significance levels are quoted in the results. With dead (frozen and thawed) nasal turbinates, lung and tracheal tissue and with an insusceptible tissue (aorta), as well as medium alone, less than 1 log₅₀ EBID₅₀ of the standard inoculum (4·6 log₅₀ EBID₅₀) could be detected in the organ culture medium after 24 h incubation.

**Washed organ cultures.** After washing three times in Hank’s balanced salt solution (Toms et al. 1976) organ culture pieces of lung and nasal turbinates (either fresh or maintained) were shaken vigorously for 2 min with organ culture medium (20 ml) and the medium
removed. After repeating the process, the pieces were distributed into dishes. Tracheas were washed intact by vigorously pipetting culture medium (20 ml) through them before they were cut into pieces and treated as described above.

_Histology._ Tissues were fixed in formal saline, embedded in paraffin wax and stained with haematoxylin and eosin or, for studies of mucus, with periodic acid–Schiff reagent (PAS) and Alcian blue.

_Staining with fluorescent antibody._ Cultures were embedded in 20% (w/v) gelatine (Burkholder, Littell & Klein, 1961), frozen in a Slee carbon dioxide freezing unit and sectioned on a cryostat. Sections were air dried and fixed in cold (4 °C) dry acetone (5 min) and stained by the indirect fluorescent antibody method. A rabbit serum against A5M was adsorbed with homogenate of ferret tissue (lung, liver, rib cage, trachea, nasal turbinate and skull), frozen in liquid nitrogen, crushed between sheets of aluminium foil and homogenized in Dulbecco PBS (A) (Toms _et al._ 1976) to minimize non-specific staining. It was used (2 drops per section) at an HI titre of 1/32 to 1/64. Fluorescein isothiocyanate (FITC) labelled, sheep anti-rabbit globulin was supplied by Wellcome Reagents Ltd, Kent. The counterstain was rhodamine RB 200 (Difco Ltd, Surrey). A Zeiss Universal Large Research microscope was used with two KP 500 exciter filters and barrier filters; either a combination of LP 520 and KP 540 (allowing fluorescence to be seen against a black background) or B47 and B55 (allowing fluorescence to be seen against orange background cells) was used.

RESULTS

_Differences in susceptibility of different regions of ferret lung_

Organ cultures of three regions of the lung were prepared: (1) cortical parenchyma, excluding all bronchi and visible bronchioles where possible; (2) hilar tissue which was largely bronchi; and (3) the peripheral lung, containing pleural mesothelium. Fresh cultures were inoculated as described in Methods and yields of A5M at 24, 48 and 72 h (Table 1) indicated that cortical parenchyma was more susceptible than other regions. Cortical parenchyma, usually from the first and second right hand lobes, was used in all experiments to avoid any variation within experiments due to differential sampling of regions of different susceptibilities. No differences in susceptibility were detected between regions of the anterior turbinates or between upper and lower trachea (the latter was used for ease of dissection).

_The effect of maintaining organ cultures of ferret lung and nasal turbinates on yields of A5M_

The results of three experiments are shown in Table 2. Virus yields at 24 h post-inoculation (p.i) from nasal turbinates were usually greater for maintained cultures than for fresh cultures but the difference was only significant (_P_ < 0.01) in one of three experiments. In contrast, the virus yield at 24 h p.i. from maintained lung was significantly greater (_P_ < 0.001) than from fresh cultures in all three experiments. Maintaining organ cultures before infection altered their susceptibility but the effect was not equal on the two tissues. In all experiments the difference in virus yields at 24 h p.i. between fresh and maintained lung was significantly greater (_P_ < 0.05) than the differences between fresh and maintained nasal turbinates (tested by the interaction component of a two way analysis of variance). Thus, in comparing maintained tissues, although virus yields at 24 h p.i. from nasal turbinates were always significantly greater than from lung cultures, the difference (5- to 6-fold) was much less than between fresh cultures (16- to 40-fold) and what might be expected from results obtained from infection _in vivo_ (Basarab & Smith, 1969). The maintenance effect was most marked on
Table 1. Virus yields from organ cultures of different regions of fresh ferret lung

<table>
<thead>
<tr>
<th>Region*</th>
<th>Virus yield (log₁₀ EBID₅₀/ml medium) post-inoculation at:†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Cortical parenchyma</td>
<td>2.2 (0.48)</td>
</tr>
<tr>
<td>Hilar tissue</td>
<td>1.3 (0.19)</td>
</tr>
<tr>
<td>Peripheral tissue</td>
<td>1.2 (0.20)</td>
</tr>
</tbody>
</table>

* See text.† Figures are the mean yields from three experiments; standard errors in parentheses.

Table 2. Replication of strain A₃M in fresh and maintained cultures of ferret nasal turbinates and lung

<table>
<thead>
<tr>
<th>Organ culture</th>
<th>Virus yield (log₁₀ EBID₅₀/ml medium) post-inoculation at:*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h in experiments</td>
</tr>
<tr>
<td>Fresh</td>
<td></td>
</tr>
<tr>
<td>Nasal turbinate</td>
<td>4.1 (0.15)</td>
</tr>
<tr>
<td>Maintained</td>
<td>4.7 (0.10)</td>
</tr>
<tr>
<td>Difference</td>
<td>0.6</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>2.7 (0.21)</td>
</tr>
<tr>
<td>Maintained</td>
<td>4.0 (0.04)</td>
</tr>
<tr>
<td>Difference</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Inoculum 4.6 log₁₀ EBID₅₀. Figures are the means of yields from at least four dishes; standard errors in parentheses.

virus yields at 24 h p.i.; at 48 h p.i. the smaller differences between fresh and maintained lung possibly reflected the fact that all replication was proceeding effectively in maintained tissue. Virus yields from nasal turbinates and lungs were also more similar at 48 h p.i. (Table 2).

Maintenance of lung and nasal turbinates for 48 h before inoculation did not produce a further increase in susceptibility. Cultures of lung maintained without antibiotics showed a maintenance effect similar to those containing antibiotics and washing cultures before inoculation produced no significant effect.

Histological changes in lung tissue produced by maintenance in organ culture

In contrast to nasal turbinates (and trachea – see later) which showed no histological difference between fresh and maintained organ cultures, the lung showed marked changes with maintenance. After 24 h a central necrotic zone contained autolysing alveolar cells; also the bronchiolar epithelia were autolyzed and desquamated and the lamina propria was vacuolated. However, the peripheral cells were normal and no different from those in fresh lung; and this was confirmed by electron microscopy. A margin of damaged cells was present at the cut edges in both fresh and maintained lung cultures.

The presence or absence of mucus did not seem to contribute to the different susceptibilities. Goblet cells were rare in the lung pieces because bronchi were excluded wherever possible and after maintenance the goblet cells of all tissues had discharged. Mucus was not detected overlying the epithelial surfaces in fresh or maintained lung and nasal turbinates either by histological procedures or by scanning electron microscopy (Kingsman, 1977).
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There was no evidence of fibroblast outgrowth or altered viable cells in lung cultures after maintenance.

Location of infected cells

Fluorescent antibody studies showed which cells became infected in fresh and maintained organ cultures of lung. Cultures were inoculated with a high dose of A₂M (6·6 log₁₀ EBID₅₀ per dish) and residual unadsorbed virus was removed after 1 h (by washing three times in 1·5 ml fresh medium) and after 7 h incubation in fresh medium, tissue sections were examined by fluorescent antibody staining. Control studies indicated that at 8 h p.i., about the end of the first cycle of replication (Toms et al. 1974), maximum intracellular staining had developed and inoculum virus could not be detected on the surface of dead (freeze-thawed) tissue nor after adsorption to live tissue for 1 h. All infected cells were confined to the periphery and the central cells in fresh lung destined to become necrotic were not infected. Alveolar type I and type II cells were the predominant cells infected in both fresh and maintained lung and the extent and distribution of fluorescence was similar in both types. The fluorescence was found mostly in single infected cells in the parenchyma scattered around the periphery but fluorescent cells were also seen in the pleural mesothelium. When similarly infected cultures were examined 24 h p.i., foci of infected cells had developed in the parenchyma of both fresh and maintained lung suggesting that initially infected single cells released infectious virus. Small foci were also observed in bronchial epithelium but foci failed to develop in pleural mesothelium indicating abortive infection which could explain the lower susceptibility of this region in culture (Table 1). Thus, infected cells were confined to the periphery of fresh and maintained organ cultures and there was no evidence in the latter for infection of any new cell types or areas.
Fig. 2. Virus (A3M) yields from organ cultures of ferret trachea inoculated (4·6 log_{10} EBID_{50}/dish) immediately after removal from animal (Δ····Δ) and after maintenance for 24 h (●—●). (a) Typical example of about half the tracheas examined; (b) typical example of the remainder.

The rapid onset of increased susceptibility and central necrosis of lung during maintenance: the effect of size of tissue pieces

Fig. 1 shows the virus yields at 24 h.p.i. from organ cultures of lung that had been maintained for 6, 12, 18 and 24 h before inoculation with A3M. Lung susceptibility increased continuously from the start of maintenance. In parallel studies uninfected cultures showed histological changes after maintenance for 6 h and central necrosis was distinct by 12 h. The
Influenza virus in ferret organ cultures

Table 3. Replication of influenza virus A/PR/8/34-A/England/939/69 clones 7a and 64d in fresh and maintained organ cultures of ferret nasal turbinates, lung and trachea

<table>
<thead>
<tr>
<th>Organ culture</th>
<th>Virus yield (log_{10}EBID_{50}/ml medium) 24 h after inoculation*</th>
<th>Clone 7a</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Clone 64d</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal turbinates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>3'8 (0·2)</td>
<td>4'8 (0·2)</td>
<td></td>
<td></td>
<td>4'7 (0·1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maintained</td>
<td>4'8 (0·1)</td>
<td>5'6 (0·1)</td>
<td></td>
<td></td>
<td>4'1 (0·2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>2'2 (0·1)</td>
<td>2'6 (0·1)</td>
<td></td>
<td></td>
<td>1'9 (0·1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maintained</td>
<td>3'7 (0·3)</td>
<td>4'O (0·2)</td>
<td></td>
<td></td>
<td>3'1 (0·2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trachea†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>0'4</td>
<td>1'5 (0·3)</td>
<td></td>
<td></td>
<td>0'4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maintained</td>
<td>1'1 (0·2)</td>
<td>2'2 (0·3)</td>
<td></td>
<td></td>
<td>0'5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Figures are the mean yields from at least four dishes; standard errors in parentheses.
† The differences in yield between fresh and maintained tracheal tissue were more marked (0·5 to 2 log_{10} EBID_{50}) at 48 h and 72 h p.i. in both experiments.

area of necrosis occupied about 50% of each section and did not increase from 12 to 24 h although autolysis and desquamation within the area increased in severity. The definite boundary of necrosis suggested deterioration might be due to limited diffusion of nutrients or oxygen to the centre. The size of the pieces of tissue in standard organ cultures was 1·5 mm³. When pieces of 1 mm³ or 2 mm³ were used both the virus yields at 24 h p.i. and the maintenance effect were similar for each size and the same as for standard cultures. However, with the smaller (1 mm³) pieces, histological change in maintained tissue was minimal; only about 10% of the total area of sections was necrotic and cell damage was not as severe as in larger pieces. Thus, despite the correlation between increased susceptibility and increasing severity of necrosis noticed previously in the standard tissue pieces, there appeared to be no causal relationship between necrosis and the maintenance effect.

Replication of A2M in fresh and maintained cultures of ferret trachea

Only about half of the tracheas examined showed a maintenance effect. In these cases virus yields from fresh cultures were poor, rarely exceeding 1·5 log_{10} EBID_{50} during 96 h p.i. but after maintenance this increased to 3·5 log_{10} EBID_{50} (Fig. 2a). In the remaining tracheas virus replicated well in both fresh and maintained cultures reaching yields of 5·0 log_{10} EBID_{50} by 96 h p.i. (Fig. 2b). These distinct replication patterns could not be predicted by any other characteristics of the ferrets and the presence of a maintenance effect in the trachea did not correspond to occasions when a small but significant effect was observed in the nasal turbinates (e.g. Expt. 1, Table 2). The maintenance effect in tracheal cultures, like that in lung cultures, was not increased by 48 h maintenance or affected by thorough washing. Histologically, maintained tracheas were similar to fresh cultures except that goblet cells had discharged all mucus and submucus glands stained less intensively. In no case was mucus overlying the epithelial surfaces and no fibroblasts were seen at the culture edges. There were no consistent differences in histology between the two types of trachea.
Clones 7a and 64d showed the same tissue specificity as A2M in fresh organ cultures of ferret respiratory tissues (Fig. 3 in Toms et al. 1976). There was also a significant increase in susceptibility after maintenance of organ cultures, which, as for A2M was more marked for lung and trachea than for nasal turbinates (Table 3). For both clones the difference in virus yield at 24 h p.i. between fresh lung nasal turbinates was about 40-fold, reducing to about ten-fold after maintenance (Table 3). A significant maintenance effect was observed in the trachea for both clones but clone 7a replicated better in fresh trachea than clone 64d (Fig. 3. in Toms et al. 1976). In contrast to the situation with the A2M strain (see Table 2), the yields of clones 7a and 64d at 48 h p.i. from fresh lung did not become more like those from maintained lung; in all cases, virus yields from maintained lung were 1 to 2 log_{10} EBID_{50} higher than those from fresh lung. Virus replication of clone 7a and clone 64 d may have been so severely limited by early events in fresh tissue that there was insufficient yield to exploit the later increased susceptibility.

DISCUSSION

With adequate care in the preparation and sampling of cultures and the use of sufficient replicates, the organ culture technique yielded results which were amenable to statistical analysis and which have indicated important biological characteristics of tissues. Former variability in virus yields from organ cultures of lung was corrected by using only the cortical parenchyma (the majority of lung tissue) and excluding less susceptible hilar and pleural tissue.

The important difference in susceptibility between organ cultures of lung and nasal turbinates was shown in virus yield at 24 h p.i. In comparisons between fresh and maintained cultures of nasal turbinates and lung, the latter showed a marked and consistent increase in virus yield at 24 h p.i. after maintenance. A significant maintenance effect with nasal turbinates was not observed in all experiments and any effect was always significantly smaller than that observed with the lung. Hence, the difference in susceptibility between lung and nasal turbinates observed in vivo (Basarab & Smith, 1969) was reflected more realistically by fresh organ cultures.

Fibroblast outgrowth which might have explained the increased susceptibility of maintained lung was not seen histologically and fluorescent antibody staining indicated no new areas of the tissue or cell type infected in maintained cultures. Also, increased susceptibility was not due to the killing of commensal bacteria by antibiotics in the medium as culture without antibiotics produced a similar maintenance effect. Poor replication in fresh lung was probably not due to loosely attached inhibitory material or to serum inhibitors released during dissection as thorough washing did not increase the virus yield. Similarly, the increased susceptibility in maintained lung was probably not due to some loosely associated promoter of virus replication formed during maintenance.

The central necrosis in lung cultures might then have caused the increased susceptibility (Chaproniere & Andrewes, 1957). Both occurred early during culture and increased in severity during the first 24 h. However, reducing the proportion and severity of necrosis by using smaller organ culture pieces had no influence on the increase in susceptibility suggesting the absence of a causal relation. Also, fluorescent antibody studies showed that the sites of virus replication were at the periphery of the organ cultures.

The presence or absence of mucus seemed unlikely to play a major role in determining
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tissue susceptibility since mucus production ceased in all maintained tissues irrespective of
the magnitude of the maintenance effect.

Two susceptibility types were represented equally in the tracheae of ferrets used in this
study. The variability of virus yields from tracheal organ cultures noted by others (Herb-
stlaier, 1970; Mostow & Tyrrell, 1973) may have been related to these different suscepti-
bilities and the maintenance effect. The maintenance effect in the trachea was not accom-
panied by any histological change other than cessation of mucus production. Because the
susceptibility type could not be predicted, tracheal cultures were less suitable than lung
cultures for analysing the basis for poor susceptibility of the lower respiratory tract
in vivo.

The marked increase in susceptibility of maintained cultures of lower respiratory tract
tissues was observed with two recombinant Hong Kong viruses. Also, in limited experiments
with the A3M strain, the maintenance effect was shown by organ cultures of mouse and
guinea pig respiratory tissue (Kingsman, 1977); and for the guinea pig, a poorly susceptible
host, the effect was noted in both nasal turbinates and lung. A maintenance effect in vitro has
also been observed by others using different viruses (Kumagi, Shimizu & Matumoto, 1958;
Frothingham, 1959; Chaproniere, 1960) and variously attributed to the acquisition of
factors not present in vivo (Chaproniere, 1960), the loss of intercellular ground substance
(Chany et al. 1966), the acquisition or uncovering of virus receptors (Holland, 1961) and
increased phagocytic activity (Gresser, Chany & Enders, 1965).

The finding that the susceptibility of some cultures change with maintenance in vitro may
help further analysis of tissue specificity within the respiratory tract in three respects. First,
a comparison of fresh organ cultures of nasal turbinates and lungs should reveal more about
the relative susceptibilities in vivo than a comparison of maintained cultures, since in
fresh cultures the differences in susceptibilities are larger and factors may be lost from main-
tained lung. Second, if the factors determining the low susceptibility of the lung in vivo are
altered by maintenance, then a comparison of fresh and maintained lung might reveal the
nature of such factors. Third, the change in susceptibility of lung occurred quickly in organ
culture, being significant at 12 h and not increasing after 24 h. Hence, the differences in
susceptibility were probably determined during the first cycle of replication, that is during the
first 6 to 8 h after inoculation. The further analysis is described in the next paper (Kingsman,
Toms & Smith, 1977).

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