Br. J. exp. Path. (1969) **50**, 612.

QUANTITATIVE STUDIES ON THE TISSUE LOCALIZATION OF INFLUENZA VIRUS IN FERRETS AFTER INTRANASAL AND INTRAVENOUS OR INTRACARDIAL INOCULATION

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Received for publication July 20, 1969

SUMMARY.—A quantitative survey of relevant organs showed that in ferrets, influenza virus localized and grew significantly only in nasal mucosa after inoculation directly into the blood stream and predominantly in that site after intranasal inoculation. In the latter case, lung and trachea became infected but to a lesser extent than nasal mucosa.

TISSUE specificity is an aspect of microbial pathogenicity about which little is known in biochemical terms. Although route of infection appears to play some role in localization of pathogenic microbes it does not seem all-important and the most likely explanations for differences in tissue susceptibility to infection are differential distribution of antimicrobial mechanisms and/or differential suitability of tissues for microbial growth (Keppie, 1964; Smith, 1968). The extra-cellular and cellular antimicrobial mechanisms of animal hosts are many and vary from tissue to tissue, but all attempts, mainly in the bacterial field, to lay the responsibility for specificity of infection unequivocally on such variations have so far failed. More success has been achieved in two investigations of the influence of nutrition on specificities of bacterial infection. Two urease-containing bacteria, *Corynebacterium renale* and *Proteus mirabilis* appear to localize in the kidneys of cattle and man because of the presence of urea in these organs. Brucellae localize, and as a result produce abortion, in the foetal placenta, fluids and chorion of susceptible animals (ox, sheep, pig, goat) because of the concentration in these organs and fluids, but not in others, of erythritol, a growth stimulant for brucellae (Keppie, 1964; Smith, 1968).

Investigations of specificities of virus infections must be conducted by an approach appropriate to the special form of parasitism. Nevertheless, the concepts outlined above provide working hypotheses for such investigations. In some instances route of infection appears important. Otherwise susceptible tissues can be protected by barriers such as the blood-brain barrier to certain blood-borne viruses that attack neurones if inoculated directly into the CNS (Bang and Luttrell, 1961) and the Küpffer cells to blood-borne influenza and myxoma viruses that attack liver parenchymal cells if injected into the bile duct (Mims, 1964).

Differences in resistance of various tissues to viral infection might be explained by differential distribution of antiviral substances such as those found in blood, macrophages and tissues (Smorodintsev, 1960; Gresser and Lang, 1966), differential production of interferon (Holland, 1964) or differential speed of stimulation of immune mechanisms (Schell, 1960). However, the importance of one or more of these factors in any example of virus specificity has yet to be proved.

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Differential suitability of cells for growth of a virus could also explain tissue specificities. To some extent this might be a question of the presence or absence of specific receptors which promote primary attachment to the cells. Although many viruses do not show marked specificity of adsorption (Fenner, 1965), specific receptors appear to be important in infection of cells with some viruses such as those of influenza and poliomyelitis (Holland, 1964; Fenner, 1965). However, even for these viruses, susceptibility has yet to be conferred on insusceptible cells by coating the latter with specific receptors. That cell receptors are not the only factors involved in tissue specificity is shown by the fact that many cells and tissues containing receptor substances to influenza virus (Papkoff, 1966) do not become infected by this virus. Specificity of virus uptake might also be influenced by compounds in the environment of cell; in tissue culture, substances in the media, including fatty acids, have stimulated virus uptake (Koch, Drén and György, 1968). On the other hand, tissue specificity may be determined by intracellular events occurring after equal penetration of virus into various cell types. Cells susceptible to a certain virus may have a biosynthetic apparatus more favourable for the growth of the virus than that of other cells. This may be a complex question of requisite enzymic activities, but differential influences of simple factors like temperature, pH (Holland, 1964) or low molecular weight nutrients might also play some role. The influence of temperature might be considered for virus localizations in the upper respiratory tract where the mucosa is at a lower temperature than most other body tissues (Willems and van der Veen, 1968). The influence of arginine on the replication of animal viruses (Becker, Olshevsky and Levitt, 1967; Rous and Schlesinger, 1967) shows that small molecules, which may vary in concentration in the metabolic pools of different cells could affect viral replication in different tissues.

To investigate these possibilities, a model virus should show marked tissue specificity in a suitable experimental animal, be easily detected and assayed and, for biochemical work on virus-host interactions *in vitro*, grow in appropriate organ cultures. The latter seem to retain their parent specificities for viruses in contrast to conventional de-differentiated tissue culture systems (Holland, 1964; Keppie, 1964). Influenza virus infection in the ferret seemed an appropriate system. In ferrets, influenza appears to take the same course as in man (Francis and Stuart-Harris, 1938) and this virus has a definite localization in the respiratory epithelium. Furthermore, the virus grows in organ cultures of ferret and human respiratory organs (Bang and Niven, 1958; Hoorn and Tyrrell, 1965).

An essential feature of any attack on the problem of tissue specificity is the identification of tissues which do not become infected either *in vivo* or *in vitro*, so that comparisons with tissues that are highly susceptible might reveal factors that either prevent infection in the former or promote it in the latter. To our knowledge no quantitative investigation exists of the distribution of influenza virus in ferrets following infection by various routes; although Laidlaw (1935) mentioned the failure to establish infection after s.c., i.v., i.p. and i.c. inoculation and Haff, Schriver, Engle and Stewart (1966) concluded from blood analyses that liver, kidney, pancreatic and adrenal functions were not affected after intranasal inoculation. This paper describes a quantitative survey of ferret organs for virus following inoculation by the intranasal route and introduction of virus into the blood stream.

MATERIALS AND METHODS

Influenza virus.—Influenza virus (A2/Moscow/1019/65, kindly supplied by Dr. H. G. Pereira as freeze dried infected allantoic fluid after 4 egg passages) was grown twice at 35° in the allantoic cavity of 12 day old embryonated eggs, for 24 hr (for seed material) and for 20 hr (for the pool of virus). The relatively short incubation periods were used to ensure as far as possible a high infectivity: total virus ratio. The pool ($10^{7\cdot8}$ EID_{50}/ml.; 5 haemagglutinating units (HAU)/ml.) which was used for most ferret inoculations was ampouled and stored in liquid nitrogen. For some experiments on direct injection of virus into the blood stream, a purified and concentrated virus suspension was prepared as follows. A second pool (*ca.* 2l.; $10^{8\cdot2}$ EID_{50}/ml., 320 HAU/ml.) was prepared from the seed. The virus was adsorbed to chicken erythrocytes, eluted at 37° with the aid of *Vibrio cholerae* neuraminidase (N.V. Philips-Duphar cholera filtrate, 1 ampoule/100 ml. Dulbecco A solution), pelleted in the ultracentrifuge (75,000 × *g*, 5 hr, at + 1°), suspended in 0·2 per cent bovine serum albumin in Dulbecco A solution (20 ml.; $10^{10\cdot2}$ EID_{50}/ml., $10^{4\cdot7}$ HAU/ml.), ampouled and stored in liquid nitrogen.

Haemagglutination, haemagglutination inhibition and infectivity assays.—Haemagglutination (HA) was carried out at room temperature with virus suspension (0·2 ml.) and 1 per cent suspension of chicken erythrocytes in Dulbecco A solution (0·2 ml.) in Perspex trays and results read after 45 min.

Haemagglutination inhibition (HI) of ferret sera was carried out as described by Schmidt and Lennette (1965) treating the sera with $NaIO_4$ and using dilutions of sera in Dulbecco A solution (0·1 ml.), virus suspension (0·1 ml., ≡ 4 HAU) and 1 per cent suspension of chicken erythrocytes in Dulbecco A solution (0·2 ml.) in HA trays.

Virus infectivity was determined by injecting 0·2 ml. of virus dilutions in Dulbecco A solution containing Crystamycin Glaxo (0·16–1·6 mg.) into the allantoic cavities of 10–13 day old chick embryos (4–5 for each 10-fold dilution), incubating for 48 hr at 35° and examining the allantoic fluids for HA activity.

Ferrets.—Adult female ferrets (600–900 g.) were obtained from the Department of Anatomy, Medical School, University of Birmingham, A. S. Roe, Little Fakenham, Norfolk, and Abbot Brothers, Thuxton, Norfolk. Ferrets were obtained from different sources because of a possible variation in susceptibility to influenza but no such variation was observed.

The sera of all ferrets showed specific HI titres $\leqslant 4$.

Intranasal inoculation.—Ferrets were anaesthetized (veterinary Nembutal Abbot, *ca.* 0·5 ml./kg. body weight, i.p.) and virus pool (0·5–1 ml., *ca.* $10^{7\cdot8}$ EID_{50}) was dropped into the nose.

Intracardial and i.v. inoculation.—Ferrets were anaesthetized with Nembutal (as above; in two cases supplemented by ether) and inoculated by heart puncture or by injection into the jugular vein. Varying amounts of virus pool ($10^{7\cdot8}$ EID_{50}/ml.) or concentrated virus pool ($10^{10\cdot2}$ EID_{50}/ml.) were injected (Table III) and the sites of injection were sterilized by application of ethanolic iodine. Aerosol formation with the virus suspension was avoided during inoculation to rule out the possibility of direct respiratory infection of the ferrets. Similarly, earlier intracardial inoculations were replaced by i.v. injections to avoid accidental puncture of a lung lobule which might take place in the former.

Intra-oesophageal inoculation.—Ferrets were anaesthetized (as above) and after section of the anterior region of the neck, the oesophagus was exposed and virus pool (1–2 ml., *ca.* 10^8 EID_{50}) inoculated in the lumen of this organ.

Investigation of organs for virus infection.—Ferrets were killed at intervals up to 5 days after inoculation by induction of air embolism whilst under Nembutal anaesthesia (as above).

Nasal mucosa lining both the nasal cavity and turbinate structures, trachea, lungs, oesophagus, CNS, muscle (from thighs), kidneys, adrenals, ovaries and oviducts, liver, bladder, spleen, eyes, heart and major blood vessels, pancreas and some clotted blood were removed aseptically with frequent changes of instruments, the organs of the thorax and nasal cavity always being dissected last.

The organs were weighed, placed in 50 per cent glycerol in Eagle's medium to make 10 or 25 per cent w/v suspensions and macerated in a Sorvall Omni-mixer with external cooling. Coarse debris were removed by light centrifugation and after adding antibiotics (Crystamycin and Mycostatin to final concentrations of 8 mg./ml. and 2000 units/ml. respectively), the supernatants were examined qualitatively for infective virus by inoculating them into 3–5 chick embryos by the allantoic route (0·2 ml. per egg) and after incubation for 48 hr at

35° examining the allantoic fluids for HA. When some or all the eggs corresponding to an organ were infected, the organ was scored + and when no eggs were infected, the organ was scored — (see Tables).

Supernatants of organ macerates showing signs of infective virus in the preliminary survey were then assayed quantitatively for virus infectivity and the results expressed as log EID_{50}/g. wet weight of organ.

As controls on the above procedures for presence of virus inhibitors in tissues and/or loss of virus titre in the preparation of macerates, relevant organs were obtained from healthy ferrets and macerated as described above in two halves, one of the halves being mixed with a known amount of influenza virus (10^4 EID_{50}). Supernatants from the uninfected halves did not produce HA in chick embryos and the virus containing halves showed no loss of virus infectivity after the processes.

RESULTS

Distribution of influenza virus in ferrets after intranasal inoculation

The results in Table I show that for at least 5 days after intranasal inoculation, influenza virus could be found in the nasal mucosa and usually in lungs, trachea and oesophagus. On the other hand, virus could not be detected in the other

TABLE I.—*Survey of Ferret Organs for Infective Influenza Virus after Intranasal Inoculation*

Ferret no.	No. of days after inoculation (*ca.* $10^{7\cdot8}$ EID_{50})	Haemagglutinating activity in chick embryos (3–5) inoculated with supernatants (0·2 ml.) of macerates of the following: Nasal mucosa	Lungs	Trachea	Oesophagus	CNS	Blood clot	Muscle	Kidneys	Adrenals	Ovaries, oviducts	Liver	Spleen	Eyes	Heart and vessels	Pancreas	Bladder
1	1	+	+	—	+	—	—	—	—	—	—	—	—	—	—	nt	nt
2	1	+	+	+	+	—	—	—	—	—	—	—	—	nt	—	—	—
3	2	+	+	+	—	—	nt	—	—	—	—	—	—	—	—	—	nt
4	2	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—
5	3	+	+	+	+	—	—	—	—	nt	nt	—	—	—	—	nt	nt
6	3	+	+	+	+	—	—	—	—	—	—	—	—	nt	—	—	—
7	4	+	—	—	—	—	—	—	—	—	—	—	—	—	—	nt	nt
8	4	+	+	—	+	—	—	—	—	—	—	—	—	—	—	—	—
9	5	+	—	—	—	—	—	—	—	—	—	—	—	nt	—	—	—

For details of methods see text: +, some or all chick embryos infected: —, no chick embryos infected: nt, not tested.

organs that were tested. The quantitative data in Table II show that in all cases the nasal mucosa contained more infective influenza virus per g. tissue than lungs, trachea and oesophagus (*cf.* Pinto, Haff and Stewart, 1969). Furthermore, in the 4 animals (ferrets 2, 4, 5 and 6) in which all 4 organs contained infective virus, the average total amounts of infective virus in lungs, trachea and oesophagus were approximately 2 per cent, 0·001 per cent and 0·1 per cent respectively of that in nasal mucosa; the average wet weights of the excised organs were 6·5 g., 0·6 g., 0·7 g. and 0·5 g. for lungs, trachea, oesophagus and nasal mucosa with turbinates respectively. There is some indication in Tables I and II that the peak of infection is on days 1–3 after inoculation.

To investigate whether the tissues of the oesophagus supported virus growth or the virus found in this organ represented spill over from the infected respiratory tract, ferrets were inoculated directly into the oesophagus to avoid concurrent respiratory infection. No infective virus could be recovered from the macerates of the oesophagus of each of 5 ferrets after 1–3 days of intra-oesophageal inoculation.

TABLE II.—*Influenza Virus Infectivity in Ferret Organs after Intranasal Inoculation*

Ferret no.*	No. of days after inoculation (*ca.* $10^{7\cdot8}$ EID_{50})	Influenza virus infectivity (log EID_{50}) in supernatants of macerates corresponding to 1 g. of the following: Nasal mucosa	Lungs	Trachea	Oesophagus
1	1	7·1	6·0	—	nt
2	1	8·3	5·3	3·4	5·3
3	2	5·5	3·5	—	—
4	2	7·2	4·3	2·7	2·4
5	3	8·2	5·2	2·9	2·9
6	3	7·2	5·3	2·7	3·2
7	4	6·2	—	—	—
8	4	6·1	3·8	—	3·7
9	5	5·2	—	—	—

Methods, see text: —, no infective virus detected: nt, not tested.
* The numbers of these ferrets correspond with those in Table I.

Distribution of influenza virus in ferrets after intracardial or i.v. inoculation

The results in Table III show that nasal mucosa became infected in 6 out of 10 ferrets receiving influenza virus directly into the blood stream. Raising the inoculum above $10^{8\cdot3}$ EID_{50} appeared to increase the frequency of nasal infection.

TABLE III.—*Survey of Ferret Organs for Infective Influenza Virus after Intracardial or i.v. Inoculation*

Ferret no.	Dose of virus (log EID_{50})*	No. of days after inoculation	Haemagglutinating activity in chick embryos (3–5) inoculated with supernatants (0·2 ml.) of macerates of the following: Nasal mucosa	Lungs	Trachea	Oesophagus	CNS	Blood clot	Muscle	Kidneys	Adrenals	Ovaries, oviducts	Liver	Spleen	Eyes	Heart and vessels	Pancreas	Bladder
1	8·3	2·5	—	—	—	—	nt	nt	nt	nt	nt	—	nt	nt	nt	nt	—	nt
2	8·3	2·5	—	—	—	—	—	—	—	—	nt	—	—	—	—	—	—	nt
3	8·3	4	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	nt
4	8·3	4	+	—	—	+	—	—	—	—	—	—	—	—	—	—	—	nt
5	8·3	4	—	—	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
6	8·6	2	+	—	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
7	8·6	2	+	—	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
8	8·6	3·5	—	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
9	10·2	3	+	+	—	—	—	—	—	—	—	—	—	—	nt	—	—	—
10	10·5	2	+	—	—	—	—	—	—	—	nt	—	—	—	nt	—	—	—

For details of methods see text: +, some or all chick embryos infected: —, no chick embryos infected: nt, not tested.
* There was no difference in results between intracardial and i.v. inoculation.

Infective virus could only be detected in lungs in one ferret and in the oesophagus in another.

Quantitative determination of virus infectivity in the nasal mucosa of the 6 infected animals (ferrets 3, 4, 6, 7, 9 and 10 in Table III) showed that they contained $10^{4.4}$, $10^{7.1}$, $10^{7.1}$, $10^{5.9}$, $10^{4.9}$ and $10^{6.3}$ EID_{50} respectively. The single samples of lung and oesophagus containing infective virus had less than 1 per cent of the virus infectivity of the corresponding nasal mucosa.

The virus recovered from some of these ferrets was confirmed to be the injected A2 strain by HI serology.

DISCUSSION

The A2 strain of influenza virus had a special affinity for the nasal mucosa in ferrets. This tissue became heavily infected after administration of the virus both intranasally and directly into the blood stream. Although the total virus content of the nasal mucosa was usually lower than the total virus introduced into the blood stream, there is little doubt that virus growth had occurred in view of the dispersion of the inoculum which would follow administration and the time lapse (2–4 days) before examination of the nasal mucosa for virus. As far as we are aware, this is the first report of respiratory infection with influenza virus in ferrets following inoculation into the blood stream, although this has been accomplished in mice with mouse adapted strains of influenza (Hamre, Appel and Loosli, 1956; Wagner, 1956).

Of the many other ferret organs examined, only lung, trachea and oesophagus contained infective virus. The amounts of virus in the trachea and oesophagus were considerably lower than in nasal mucosa or lungs and the virus found in oesophagus represented spill-over from the infected respiratory tract as indicated by the experiments of intraoesophageal inoculation.

As a preliminary to studies on the chemical basis of tissue specificity, ferret tissues susceptible and insusceptible to influenza infection by two routes *in vivo* have been identified. The corresponding behaviour of the virus in selected organ cultures will be described later.

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