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THE LOCALIZATION OF INFLUENZA VIRUS: MINIMAL INFECTIOUS DOSE DETERMINATIONS AND SINGLE CYCLE KINETIC STUDIES ON ORGAN CULTURES OF RESPIRATORY AND OTHER FERRET TISSUES

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Summary.—A comprehensive survey of ferret tissues in organ culture showed that influenza virus (A/Moscow/1019/65 (H_2N_2)) can infect bladder, uterus, oviduct and conjunctiva in addition to respiratory tissues. Alimentary tract tissues were insusceptible, except oesophagus and pharynx which were infected at high inocula. Muscle, reticuloendothelial tissues, blood vessels and kidney were also insusceptible.

Minimal infectious dose determinations on the susceptible tissues showed significant differences in the ease of initiation of infection in the order nasal turbinates > bladder > uterus > trachea > lung > conjunctiva > oviduct > oesophagus. On the other hand, the susceptible tissues were similar with respect to the kinetics of virus replication over one infection cycle, new virus appearing from all tissues at about the same time (5–6 hours) after inoculation.

Large inocula of virus disappeared very quickly from the blood stream of ferrets *in vivo*, yet *in vitro* ferret blood could not inactivate large quantities of virus although it inhibited smaller quantities.

The results are discussed in relation to the degree of confinement of influenza infection to the respiratory tract.

FOLLOWING intranasal inoculations of an A(H_2N_2) strain of influenza virus into ferrets, of 16 tissues examined only nasal turbinates, lung, trachea and oesophagus contained significant quantities of virus, and of these nasal turbinates were most susceptible to infection, containing over 95% of the total virus recovered (Basarab and Smith, 1969). Similarly, after inoculation into the blood stream only the nasal turbinates were consistently infected with occasional isolations from lung and oesophagus (Basarab and Smith, 1969). In organ cultures of a limited number of tissues (nasal turbinates, trachea, lung, oesophagus, aorta, bladder and oviduct, actually the distal end of the uterine horn) virus growth patterns paralleled those *in vivo* for the respiratory tract tissues (Basarab and Smith, 1970). Lower, and later peak, titres of virus were obtained in lung and trachea cultures compared with those from nasal turbinates. Aorta and oesophagus were insusceptible to infection with the inoculum used (10^4 egg infective $dose_{50}$; Basarab and Smith, 1970; Gould *et al.*, 1972). However, bladder and uterus seemed at least as susceptible to infection as some respiratory tract tissues, a fact confirmed by infecting them *in vivo* by direct urogenital inoculation (Basarab and Smith, 1970). The different patterns of infection of the susceptible tissues in organ culture and the animal

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experiments clearly indicated differences in susceptibility; in both cases virus production was monitored over several days and thus involved more than one replication cycle.

In this paper we first extended the long-term organ culture studies to the original and additional extra-respiratory tissues to see if any became susceptible when higher virus inocula were used. Also, the response of the susceptible tissues to increased virus dose has been examined to see if differences in patterns of virus growth between the tissues were dose dependent. Then we investigated two of the possible explanations for the different patterns of infection occurring in the susceptible tissues: (1) difference in the efficiency with which virus reaches and initiates infection in the susceptible cells, the number and availability of which may vary from tissue to tissue; (2) differences in the kinetics of the virus replication cycle in the individual susceptible cells of the different tissues. The former has been examined by comparing minimal infectious doses for the various susceptible tissues in organ culture. The second has been examined by determining and comparing the curves for a single replicative cycle in each tissue. Finally, the behaviour of bloodborne virus was examined *in vivo* and *in vitro* to shed light on the inability of such virus to infect potentially susceptible tissues other than nasal mucosa. The results are discussed in relation to the degree of confinement of influenza virus to the respiratory tract tissues in ferrets and in man.

MATERIALS AND METHODS

Influenza virus.—The strain A/Moscow/1019/65 (H_2N_2), and the preparation of virus stocks were described by Basarab and Smith (1969). High titre pools were prepared by the method of Gould *et al.* (1972). The mouse neurotropic NWS strain was supplied by Dr D. Hobson, Department of Microbiology, University of Liverpool.

Infectivity assays.—These were performed by the egg bit technique of Fazekas de St Groth and White (1958) which was compared with titrations in 10-day old embryonated eggs (Basarab and Smith, 1969) for each virus stock at intervals during its use. Titres of $6{\cdot}8 \pm 0{\cdot}2$ $\log_{10}$ $EBID_{50}$ (50% egg bit infectious dose) and $8{\cdot}3 \pm 0{\cdot}5$ $\log_{10}$ EID_{50} (50% egg infectious dose) were obtained from 28 independent titrations in egg bits and 10 independent titrations in eggs of a single virus pool.

Haemagglutination (HA) and haemagglutination inhibition (HI) assays.—These were described by Basarab and Smith (1969) using a 1% (v/v) suspension of human "type 0" erythrocytes.

Ferrets.—These were obtained from A. S. Roe, Norfolk and were as described by Basarab and Smith (1969).

Collection of blood.—Ferrets were anaesthetized (veterinary Nembutal, Abbott *ca.* 0·5 ml/kg body weight, i.p.) and bled out by heart puncture into heparin (1·0 i.u./ml) after first taking a 2 ml sample for HI tests (Basarab and Smith, 1969).

Measurement of virus inactivation in ferret blood.—Ferret blood (2·7 ml) was mixed with the virus suspension (0·3 ml; low, $10^{6{\cdot}3}$ $EBID_{50}$/ml, or high, $10^{9{\cdot}4}$ $EBID_{50}$/ml, titre pools). The mixture was held at 37° for 24 hours and at intervals samples were taken (0·2 ml). The virus in whole blood, plasma and cells was titrated; the latter were separated by centrifugation in the cold (1500 *g*, 10 min). Each sample was mixed with Dulbecco A solution with 1% w/v bovine serum albumin (1·8 ml) and frozen at −70° until required for titration.

Intracardial inoculation.—This was described by Basarab and Smith (1969).

Virus isolation from ferret blood and tissues.—Ferrets inoculated with influenza virus intracardially were held under anaesthesia for up to 2 hours. At intervals blood samples (0·2 ml) were collected into heparin from the jugular vein by the method of Bergman, Lodmell and Hadlow (1972). After 2 hours a final blood sample was taken by cardiac puncture and the animals were killed by induction of air embolism. Organs were collected, weighed and macerated in a Sorvall omnimixer by the method of Basarab and Smith (1969) but using Dulbecco A solution with 1% w/v bovine serum albumin instead of Eagle's medium with

glycerol. Maceration in the new medium did not significantly affect infectivity titres. Macerates and blood samples were titrated for infectivity in egg bits. Inhibitors in the samples prevented the detection of influenza virus in dilutions less than 10^{-2}.

As a more stringent test for the presence of virus in some ferrets, organ cultures were set up from tissues susceptible to influenza and the medium monitored for virus over 5 days by titration in egg bits.

Organ cultures.—These were prepared by the method of Hoorn and Tyrrell (1965) with the medium of Basarab and Smith (1970) but omitting the bovine serum albumin. Viability was checked at the end of experiments, and sometimes in parallel cultures during experiments, by the tetrazolium reaction (Hershey, Cruickshank and Mullins, 1958) or by staining with neutral red. Any cilial activity of explants was checked. Explants were also examined histologically after fixation in buffered formol saline or preparation in a cryostat and staining with haematoxylin and eosin. Explants of nasal turbinate, trachea, lung, pharynx, oesophagus, bladder, uterus, oviduct, vagina, conjunctiva, heart, aorta, diaphragm, thigh muscle and salivary gland showed no substantial necrotic changes over 96 hours in culture. Stomach, jejenum, colon, liver, kidney and ovary showed marked necrosis in 96 hours but not at 24 hours while lymph node and spleen showed significant changes even after this short time. In Table I, which relates to virus production over 96 hours, these tissues appeared insusceptible. However, they were examined for capacity to support virus replication in short-term experiments over 8 hours (following 20 hours preliminary incubation) with high virus inocula (Table III) before being designated as insusceptible.

TABLE I.—*Susceptibility of Ferret Organ Cultures to Infection with Influenza Virus*

Organ culture	Inoculum	Susceptibility
Nasal turbinates anterior	4·6	+
Nasal turbinates posterior	4·6	+
Trachea	4·4	+
Lung	4·4	+
Pharynx	4·6	+
Oesophagus	4·4	+
Stomach	6·6	−
Jejunum	6·6	−
Colon	6·6	−
Salivary gland	4·6	−*
Liver	4·6	−
Heart (left ventricle wall)	4·6	−
Aorta	4·4	−
Diaphragm	4·8	−
Thigh muscle	4·6	−
Lymph node	4·6	−
Spleen	4·6	−
Kidney	4·6	−
Bladder	4·4	+
Uterus	4·4	+
Oviduct	3·4	+
Ovary	4·6	−
Vagina	4·6	−*
Conjunctiva	4·5	+

At least 3 experiments were conducted on tissues from 3 separate ferrets. +, at least 2 of media samples taken at 48, 72 and 96 hours contained significant infectivity (greater than 100 $EBID_{50}$) in all experiments. −, no significant infectivity detected in any sample in all experiments.

* In one experiment significant infectivity was detected.

Unless otherwise stated, inoculation of the organ cultures and their follow-up were as described by Basarab and Smith (1970). In the investigation of susceptibility of the various tissues (Table I), experimental and control (virus inoculum but not tissue pieces) dishes were incubated for 4 days, the medium being replaced and titrated every 24 hours. Virus could not be detected (less than 10 $EBID_{50}$) in the control dishes at 48 hours. Hence significant infectivity was 100 $EBID_{50}$ in media taken 48 hours and after. Tissues were designated susceptible if at least 2 of the media samples taken at 48, 72, and 96 hours had significant infectivity in at least 3 experiments with tissues from 3 different ferrets.

Determination of minimal infectious doses.—This was done by an end point assay and experiments were repeated at least 3 times, each time with tissue from one or more different ferrets. In each experiment 4 organ culture dishes containing 4 pieces of tissue were inoculated with each ten-fold dilution of the virus stock (titre, $10^{8\cdot2}$ $EBID_{50}$/ml). To promote optimum efficiency of infection, adsorption of the virus inoculum was conducted in a small volume as follows. After overnight incubation at 37° the 4 tissue pieces from each culture dish were placed in the well of a microtitration plate (Flow Laboratories Ltd) with 0·05 ml of the appropriate virus dilution in cold medium. After 4 hours at 4° the tissue pieces were transferred back to their original scratched dishes with fresh medium (1·5 ml). Control dishes contained medium (1·5 ml) and the appropriate virus dilution (0·05 ml). The dishes were then incubated for 4 days in the normal manner, the medium being replaced and titrated for infectivity every 24 hours. Virus could not be detected (less than 10 $EBID_{50}$) in control dishes at 48 hours, even in those with the lowest dilutions of virus suspensions. Hence in experimental samples 100 $EBID_{50}$ or more of virus detected 48 and 96 hours after inoculation showed that significant infection had occurred. The minimal 50% infectious dose (MID_{50}) of the virus for each tissue was calculated from the results for all 4 dishes at each dilution by the moving averages method (Thompson, 1947). The mean MID_{50}'s expressed in $EBID_{50}$ for the 3 or more determinations on each tissue, are quoted in Table II.

TABLE II.—*Minimal Infectious Doses (MID_{50}) of Susceptible Ferret Tissues in Organ Culture*

Tissue	MID_{50} (log $EBID_{50}$)
Nasal turbinate	1·2 (0·4)
Bladder	1·6 (0·7)
Uterus	1·8 (0·4)
Trachea	2·8 (0·2)
Lung	2·8 (0·4)
Conjunctiva	3·0 (0·2)
Oviduct	3·0 (0·5)
Oesophagus	⩾4·2 (—)

The values (standard error in brackets) are the means for at least 3 experiments.

Single cycle growth studies.—In these short-term experiments cultures were held in bijou bottles and not in the conventional culture dishes. Explants (normal size; about 50) of each tissue were placed in bijou bottles with medium (1 ml) containing a high virus inoculum (10^7 $EBID_{50}$). The explants in each bijou were then treated as follows: After 30 min in a water bath at 37° with occasional gentle shaking, the inoculum was removed and the explants washed 3 times in warm medium (5 ml). At this point 6 explants were removed, macerated and titrated for infectivity to determine adsorbed virus. The remaining explants were then transferred to a fresh bijou and an appropriate dilution (1 ml) of a solution of anti-influenza rabbit γ-globulin (Sweet, Stephen and Smith (1973); HI titre, 1/32,000) was added to neutralize extracellular virus. The amount of antibody used (1/5 dilution of the preparation) was approximately double the minimum shown in preliminary experiments to neutralize external virus that had been adsorbed as described above on nasal turbinate explants. After 30 min at 37° in the water bath to effect neutralization, the antibody solution was removed and the explants were washed 3 times in warm medium (5 ml). The explants were again transferred to a fresh bijou and goat anti-rabbit gamma-globulin (1 ml; Cappel Laboratories), was added. After a further 30 min at 37° the goat serum was removed and the explants washed 3 times with warm medium (5 ml). At this point the remaining pieces were divided into aliquots of 6 explants. These were transferred to fresh bijoux containing warm medium (1 ml). One (6 explants) was taken immediately and macerated and titrated for infectivity to check that all virus had been neutralized. The remaining bijoux were placed at 37° and one aliquot (6 pieces) was taken for assay after a further 2, 3, 4, 5, 6 and 22 hours. In this complicated procedure transfer of explants from bijoux to bijoux was achieved by sucking the tissue bits in the medium of the terminal wash into a glass tube (0·5 mm diameter), excess washing fluid being easily removed after transfer with a fine Pasteur pipette. In this way minimal damage to the explants was ensured. The whole adsorption and neutralization

procedure took between 2 and 2½ hours to complete, depending on the number of tissues under study. Hence the total time elapsing to the taking of the first sample was 2½ + 2, 4½ hours (see Fig. 3, 4). Tissue pieces (6) and supernatant fluid were assayed separately, the former being macerated in medium (1·5 ml) in a Sorvall Omnimixer with external cooling. To each an equal volume of organ culture medium with bovine serum albumin (2·5% w/v) was added and the samples were stored at − 70° for subsequent infectivity titrations. Figure 4 represents the average titrations of 4 or more similar experiments on each tissue.

RESULTS

Survey of ferret tissues for susceptibility to infection with influenza virus

In addition to respiratory tract tissues, bladder uterus and oviduct (Basarab and Smith, 1970), conjunctiva, oesophagus and pharynx consistently supported

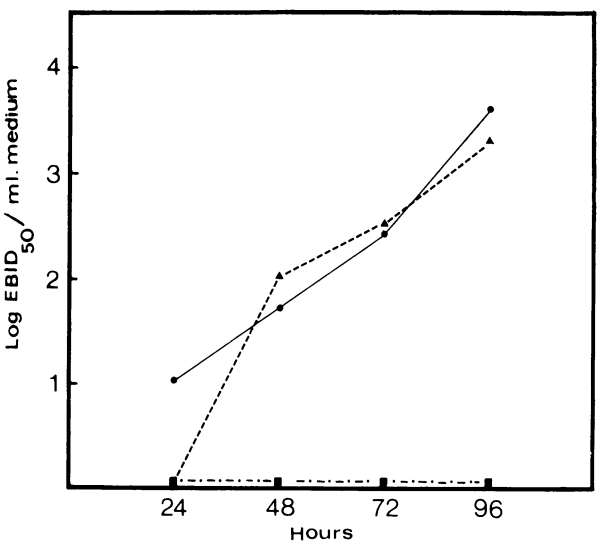

FIG. 1.—Virus released into the media of organ cultures of oesophagus with 3 inocula; ■–·–■, 10^4 $EBID_{50}$; ●– –●, 10^5 $EBID_{50}$; ▲– –▲ 10^6 $EBID_{50}$.

replication of influenza virus (Table I). The failure of Basarab and Smith (1970) and Gould *et al.* (1972), to detect significant infection of the oesophagus was due to using a low virus inoculum (10^4 EID_{50} ≡ approximately 10^3 $EBID_{50}$). An inoculum of 10^5 $EBID_{50}$ was needed to yield significant quantities of virus from oesophageal cultures (Fig. 1). Figure 2 shows the effect of raising the virus inoculum on virus production by the susceptible tissues. With the small inoculum ($10^{4\cdot6}$ $EBID_{50}$) only nasal turbinates showed a peak virus yield at 24 hours, the remainder taking 48 hours or longer to reach their peaks. The higher inoculum (10^6 $EBID_{50}$) produced a reduction in infectivity yields from nasal turbinates, bladder, uterus and lung cultures and all showed peak virus yields at 24 hours; thus the differences in the pattern of virus production observed at lower inocula tended to disappear. In contrast, inoculum size did not have a striking effect on virus production by trachea (Fig. 2) or by oesophagus (Fig. 1, 2) once the threshold inoculum was exceeded.

All other tissues tested failed to support detectable virus replication; these

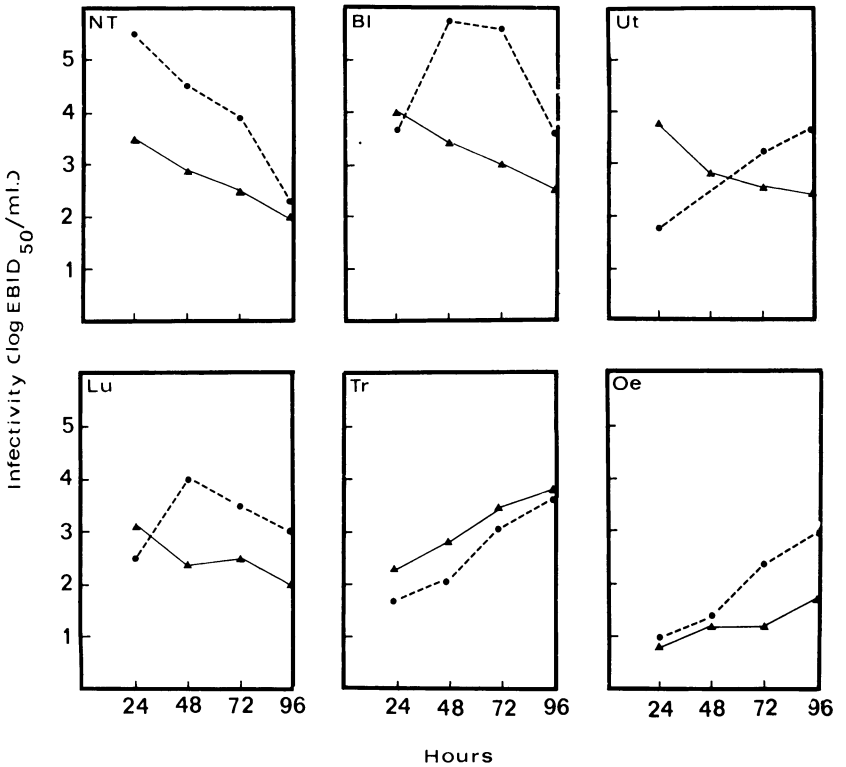

FIG. 2.—Effect of increasing virus inoculum on virus yields in media from organ cultures of susceptible tissues (NT, nasal turbinates; Bl, bladder; Ut, uterus; Lu, lung; Tr, trachea; Oe, oesophagus). ▲——▲, inoculum 10^6 $EBID_{50}$; ●– – –●, inoculum $10^{4.6}$ $EBID_{50}$.

included representatives of the alimentary tract, the reticuloendothelial system, endothelial and mesothelial surfaces and glandular tissue as well as kidney and muscle. However, these experiments were conducted over 96 hours and by this time some tissues (stomach, jejunum, colon, liver, lymph node, spleen, kidney and ovary) showed marked necrosis. Hence their susceptibilities were checked in short-term (8 hours) experiments with high inocula (see Table III). The specific nature of the insusceptibility of some tissues to the A_2 strain of influenza virus was underlined by the fact that these tissues supported the replication of the neurovirulent NWS strain of influenza virus. Thus virus titres in excess of 100 EID_{50} were detected in cultures of aorta, spleen, liver and kidney between 48 and 96 hours after inoculation with $10^{5.6}$ EID_{50} of this strain but not in control cultures.

Minimal infectious doses of organ cultures of the susceptible ferret tissues

Minimal infectious doses of the susceptible tissues (Table II) indicated that initial infection of nasal mucosa with influenza virus occurred more readily than in other respiratory tissues, trachea and lung. Furthermore, bladder and uterus became infected more easily than trachea and lung, and bladder even approached nasal mucosa in its affinity for influenzal infection. Conjunctiva and oviduct approximated to trachea and lung in ease of infection.

TABLE III.—*The Inability of Ferret Aorta, Kidney, Spleen, Liver, Lymph Node, Stomach, Jejunum, Colon and Ovary Organ Cultures to Support a Single Cycle of Influenza Virus Replication*

Organ culture	Virus adsorbed to tissue 30 min after inoculation	Virus ($\log_{10}$ $EBID_{50}$/ml) in whole* cultures at		
		2 hours	8 hours	24 hours
Aorta	4·2†	<1·0	<0·5	<0·5
Kidney	4·2	<1·8	<0·7	<0·5
Spleen	3·5	<0·7	<0·7	<0·5
Liver	3·7	<0·5	<0·5	<0·5
Lymph node	4·0	<0·5	<0·5	<0·5
Stomach	3·9	<0·6	<0·5	<0·5
Jejunum	3·7	<0·5	<0·5	<0·5
Colon	3·7	<0·5	<0·5	<0·5
Ovary	4·3	<0·6	<0·6	<0·5
Anterior nasal turbinate	4·0	<0·9	3·2	4·5

Inoculum $10^{7.4}$ $EBID_{50}$ influenza virus.
* Virus determinations were made on a macerate of the tissue pieces in the supernatant medium.
† Standard error +/− 0·2 $\log_{10}$ $EBID_{50}$.

Single cycle kinetic studies on organ cultures of the susceptible ferret tissues

In these short-term experiments masking of early virus production by residual virus inoculum was overcome by rapid neutralization of the latter with anti-influenza rabbit γ-globulin; the amount used was twice that indicated by preliminary titrations to be needed to neutralize virus adsorbed on the most susceptible tissue nasal turbinate. Unfortunately, some antibody was retained on the tissues despite thorough washing and subsequently it reduced the measured yield of virus;

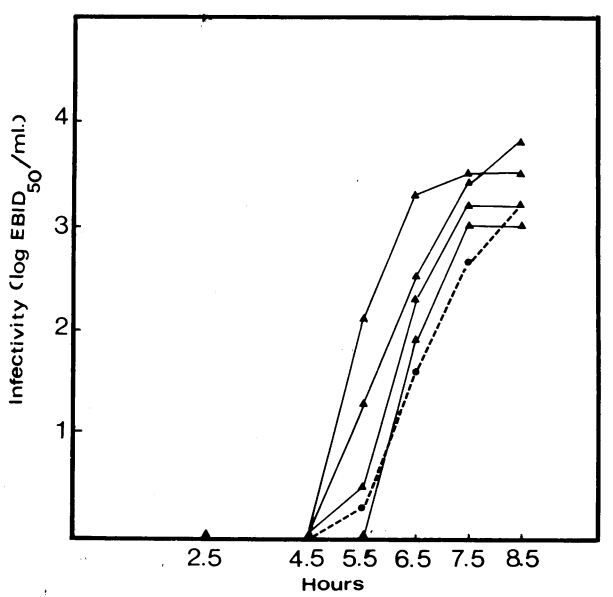

FIG. 3.—Virus released into the medium in short-term, single cycle infection experiments in nasal turbinate cultures. ▲——▲, 4 different experiments with tissues from different ferrets with antibody neutralized by goat anti γ-globulin; ● · · · · ●, a similar experiment but without addition of anti γ-globulin.

treatment with goat anti γ-globulin reversed this effect possibly by inactivating the residual antibody (Fig. 3). Four identical infection experiments (Fig. 3) with nasal mucosal organ cultures from different ferrets showed that the single cycle kinetics were repeatable with a variation of only $\pm$ 0·5 log at the maximum titre (8 hours) although, as might be expected, variation was more ($\pm$ 1 log) at the time of first appearance of virus (5·5 hours).

Average curves (Fig. 4) were prepared from 4 experiments in which early virus production by all the recognized susceptible tissues was measured. Not only was virus released into the medium represented but also virus was present in the tissue pieces. With the exception of the oesophagus, the virus production curves were similar despite the differing susceptibilities of the tissues. New cell associated virus first appeared at 5·5 hours and reached peak titres at 7·5–8·5 hours. Virus

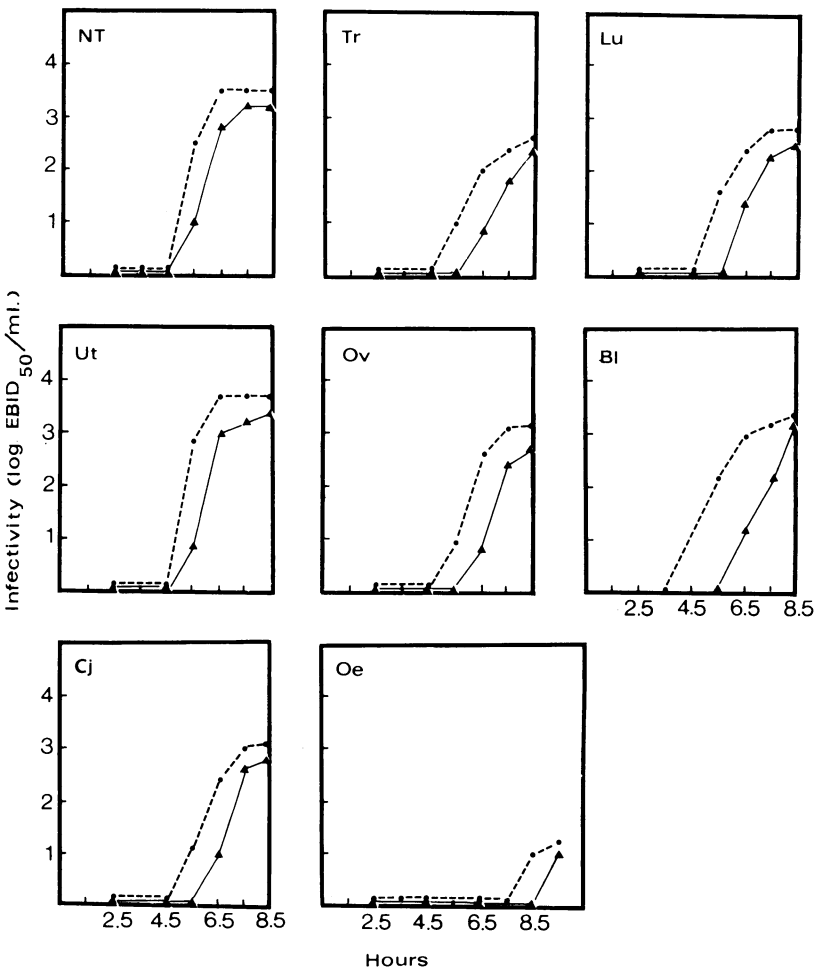

FIG. 4.—Virus released into the medium ▲——▲ and cell associated virus ●– –● in single cycle infection experiments with organ cultures of nasal turbinates (NT), trachea (Tr), lung (Lu), uterus (Ut), oviduct (Ov), bladder (Bl), conjunctiva (Cj) and oesophagus (Oe).

was released into the medium within an hour of its detection in the tissue pieces. Peak titres in nasal turbinate, bladder and uterus were about 1 log $EBID_{50}$ above those in lung and tracheal cultures, with those in oviduct and conjunctiva occupying an intermediate level. New virus was not detectable in the oesophageal cultures until *ca.* 8 hours after infection and peak titres were markedly below those in other tissues.

In these experiments the amount of virus absorbed to the various tissues was measured. This was done 30 minutes after treatment with a high virus inoculum (10^7 $EBID_{50}$) and after washing but before neutralization of external virus by antibody. The following figures ($\log_{10}$ $EBID_{50}$ with standard errors of *ca.* 0·2) were obtained; nasal turbinates 4·0; bladder 3·6; uterus 3·8; trachea 3·9; lung 4·4; conjunctiva 4·1; oviduct 4·0 and oesophagus 3·9. Table III summarizes the results of experiments conducted in a similar fashion to those with the susceptible tissues, *i.e.* with neutralization of any residual virus inoculum with antibody, but using tissues that have been designated insusceptible in the long-term experiments. Although it was difficult reliably to neutralize all residual inoculum, virus was only isolated, if at all, in very small quantities from these tissues, thus confirming their insusceptibility. Nevertheless, the tissues adsorbed similar amounts of virus as the susceptible tissues.

The rapid disappearance of high levels of infectivity from ferret blood in vivo *but not* in vitro

Table IV summarizes the recovery of infective virus from various tissues 2 hours after intracardial injection of a large quantity of virus ($10^{9.4}$ $EBID_{50}$ in

TABLE IV.—*Isolation of Virus from a Ferret 2 Hours after Intracardial Inoculation with* $10^{9.4}$ *EBID*$_{50}$ *of Influenza Virus*

Sample	Infectivity ($\log_{10}$ $EBID_{50}$/g)
Whole blood*	—
Peritoneal exudate	—
Heart	<3·0‡
Aorta	<3·0
Liver	<3·0
Kidney	—
Spleen	3·0†
Lymph node (cervical)	—
Lung	—
Nasal turbinate	—
Salivary glands	—
Bladder	—
Uterus	—
Urine	—

— No detectable virus (< 10 $EBID_{50}$).

* Virus titres ($\log_{10}$ $EBID_{50}$/g) detected at 5 min, 15 min and 30 min post-inoculation were 4·0, 2·6, and — respectively.

† Standard error 0·2 $\log_{10}$ $EBID_{50}$.

‡ Owing to the presence of inhibitors of virus haemagglutination in blood and tissue macerates virus titres of < 3·0 $\log_{10}$ $EBID_{50}$/g could not be estimated accurately.

1·5 ml Dulbecco A solution). Five minutes after the injection a blood sample contained $10^{4.0}$ $EBID_{50}$ of virus. After 2 hours when the animal was killed infectious virus could not be detected in the blood and only minute amounts

compared with the inoculum were recovered from the heart, aorta and liver; a significant but small infectivity was detected in the spleen but virus was not detected in the most susceptible tissue, nasal turbinates. Nor was virus isolated over 5 days from organ cultures of nasal turbinates, lung, uterus and bladder prepared from the inoculated animal.

Such a dramatic reduction of infectivity did not occur in a mixture of blood and high titre virus *in vitro* (Fig. 5a). Most of the virus remained free in the plasma

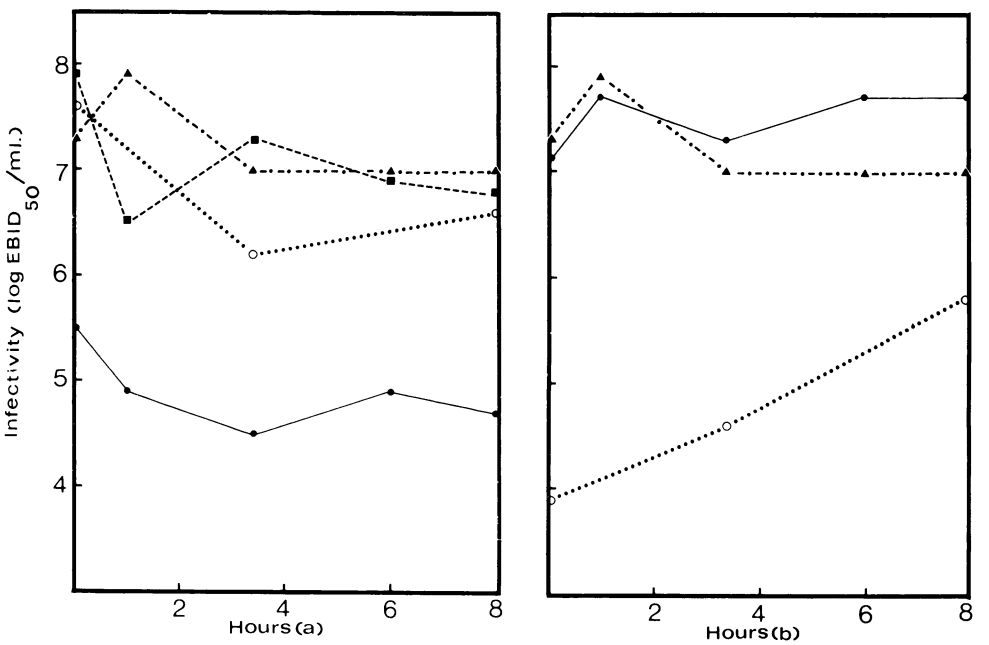

FIG. 5.—Stability of influenza virus *in vitro*. (a) in whole ferret blood: □- - -□, whole blood; △-·-△, control in Dulbecco A solution; ○ · · · · ○, plasma; ●——●, blood cells. (b) in suspension of washed blood cells in Dulbecco A solution: ●——●, blood cells; ○ · · · · ○, supernatant; △-·-△, control.

and was not adsorbed on to the red or white blood cells. In contrast, when the virus was mixed with a corresponding amount of washed blood cells (in Dulbecco A solution containing heparin 1 iu/ml) while the infectivity was not appreciably reduced, it remained associated with the cells and only 10% was found in the plasma even after 8 hours (Fig. 5b).

Use of low titre virus ($10^{6\cdot5}$ $EBID_{50}$) showed that some inactivation occurred in whole blood *in vitro*. In a representative experiment the virus titre in whole blood was reduced in 1 hour at 37° from $10^{6\cdot5}$ $EBID_{50}$ to undetectable levels (< 10 $EBID_{50}$). The control virus suspension in Dulbecco A solution with 1 iu heparin per ml was unaffected in this short period.

DISCUSSION

The comprehensive survey of ferret tissues in organ culture showed that only a few of the extra-respiratory tract tissues tested were susceptible to influenza virus

infection and of these, bladder and uterine horn with attached oviduct had been recognized previously (Basarab and Smith, 1970). Conjunctiva was an additional susceptible tissue and oesophagus and pharynx could be infected by high virus inocula. The rest of the tissues examined, which included mucosae from the lower alimentary tract, kidney and reticuloendothelial tissues, were insusceptible to infection, even when some of them (Table III) received very high virus inocula and the cultures were examined for fresh virus after one cycle. The virus used was a typical A(H_2N_2) strain and some of the tissues insusceptible to infection with this strain might prove susceptible to infection with more virulent A strains or other antigenic types. Certainly the neurotropic NWS strain replicated in aorta, spleen, liver and kidney. However, this is a specially selected and hardly typical influenza virus which is pantropic in the mouse (Wagner, 1955). The results with NWS highlight the specificity of the insusceptibility of the cultures to the A (H_2N_2) strain.

The infection of ferret urogenital tract tissues recalled reports of the presence of influenza virus in human urine (Zakstelskaya, 1953; Davenport, 1961) and prompted a successful demonstration of influenza virus replication in human endometrial but not myometrial organ cultures (Rosztoczy, Toms and Smith, 1973). These results, together with previous reports of influenza virus replication in foetal tissues (Hoorn and Tyrrell, 1969; Yawn, Pyetta and Mehsen-Joseph, 1971) are relevant to recent interest in possible congenital effects of influenza (Fedrick and Alberman, 1972; Wynne-Griffiths *et al.*, 1972). The susceptibility of ferret conjunctiva to infection suggests that conjestion of conjunctivae in human infection (Hoyle, 1968) may be due to virus replication at this site rather than to a secondary effect.

A comparison of the minimal infectious doses clearly indicated differences in the efficiency with which the virus reaches and initiates infection of cells within the various susceptible tissues. Efficiency of infection was highest in nasal turbinates, lower in uterus and bladder and lower still in trachea, lung, conjunctiva and oviduct. As expected (Gould *et al.*, 1972), the efficiency of virus infection of susceptible cells in the oesophagus was lowest of all.

With the exception of oesophagus, the kinetics of virus production over one cycle were essentially the same for all tissues despite their varying susceptibility. This was both surprising and interesting, suggesting that whatever the tissue intracellular production of new virus occurs by a similar process. New virus may have been produced from the oesophagus at the same time (*ca.* 5 hours) as that from the other tissues but the amount would have been too small to detect.

In the single cycle kinetic experiments the amounts of virus adsorbed by all the susceptible tissues were similar. Also, the insusceptible tissues (Table III) adsorbed similar amounts ($10^{4\cdot0}$ $EBID_{50}$) of virus. These results emphasize that there are many insusceptible cells in organ cultures that can adsorb virus non-specifically. These may be on the cut edges of the tissue pieces and have no relevance to the situation *in vivo*. Clearly the important parameter, the amount of virus adsorbed by the susceptible cells, is not attainable by these simple experiments.

The results raise two main questions. First, why is localization of infection in the respiratory tract the general pattern of influenza when there are equally, if not more, susceptible tissues having the same virus replication kinetics elsewhere in the animal? Second, what are the reasons for the different susceptibilities of the respiratory tract tissues which fit with the fact that influenza is primarily an upper respiratory tract infection?

Obviously route of infection, coupled with the high susceptibility of nasal mucosa and the adequate susceptibilities of the other respiratory tissues, explain the initial localization. In addition, however, our experiments on blood infection suggest some barrier prevents spread to the susceptible urogenital tissues. Very large quantities of infective virus disappeared from the blood stream within 30 minutes of direct inoculation and only minute amounts were found in the tissues after 2 hours, the most being in the spleen. Two to 3 days after such inoculation neither the bladder nor uterus were infected (Basarab and Smith, 1969). The disappearance of the large amount of infective virus was probably not due to direct inactivation by blood. When incubated with ferret blood *in vitro* large quantities of virus were not inactivated (and remained free in the plasma in contrast to a strong adsorption to *washed* blood cells, Fig. 5b). On the other hand, smaller quantities of virus were inactivated *in vitro*, probably by the β inhibitors of ferret blood (Burnet and McCrea, 1964) and if small quantities of virus escaped from respiratory tract these inhibitors could constitute an effective barrier. Vascular endothelium appeared insusceptible in organ culture at least to this strain of influenza virus and thus it might provide a barrier to infection of some tissues. But the reticuloendothelial system may play the major role in rapidly reducing any viraemia and thus the potential inoculum for a susceptible tissue. Influenza virus seems to quickly adsorb to and penetrate into phagocytic cells (Davenport, 1961; Sawyer, 1969). When large quantities of virus were inoculated intracardially only the spleen had significant amounts of infectious virus after 2 hours. Also, virus was detected by us in the cervical lymph nodes but not in the blood 24 and 48 hours after intranasal inoculation of virus. Further investigation of the barrier must involve methods of detecting non-infectious virus after blood inoculation of large quantities of infectious virus.

As no differences in replication kinetics of the virus were detected in the susceptible respiratory tract tissues, susceptibility is probably determined either at initiation of infection or at the release of infectious virus. The different minimal infectious doses of the various tissues clearly indicate differences in ease of initiation of infection. Factors determining the different MID_{50}s could exist at the tissue level or at the level of the individual susceptible cells.

Nasal turbinates may contain a higher proportion of available susceptible cells which would allow greater absolute amounts of virus to enter the tissue for the first cycle, also providing more cells for infection at later cycles. Availability of the susceptible cells as well as their numbers should be stressed. Clearly, from electron microscopy of nasal turbinates and oesophageal infection (Gould *et al.*, 1972) virus can contact the susceptible cells in the turbinates easily but may be hindered in contacting any susceptible cells in the oesophageal surface by a thick overlay of cell debris. Similar structural features might obscure susceptible cells in trachea and lung and the curves of virus production in the long-term experiments at low and high inocula (Fig. 2) are interesting in this respect. At high inocula any differences occurring between nasal turbinates, bladder and uterus with low inocula disappeared. Although this picture was complicated by auto-interference observed in other systems with high inocula (von Magnus, 1946), it suggests that a similar number of susceptible cells in the 3 tissues are available to the higher inoculum. The lung cultures showed a similar pattern of virus production with high inocula, including the auto-interference, but the peak titres tended to be lower than those for nasal turbinates, suggesting that the number of lung cells available for infection

may be lower. With trachea (and oesophagus) an increasing inoculum over the MID_{50} has little effect on the pattern of infection or virus yields (Fig. 1, 2), indicating that the majority of the susceptible cells are not available to infection by the inoculum and that those that are, are saturated even with the lower inoculum. Infection appears to progress in subsequent cycles under some structural hindrance, as discussed above.

At the cell level, differences in ease of initiation of infection may relate to the amounts of virus required to start the process and differences in amounts of virus absorbing to or penetrating into susceptible cells.

There remains the possibility, which has not been investigated here, that differences in the numbers of infectious virions released from infected cells of different tissues may play a role in their susceptibility as well as variations in their ease of transmission to other susceptible cells. Such factors as differential interferon production or production of non-infectious interfering virus may have an effect in this area. Such effects might not become noticeable until after several cycles of virus replication. These possibilities will be the subjects of future work.

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