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THERMAL STABILITY AND INTERACTION WITH FERRET INFLAMMATORY EXUDATES OF TWO CLONES OF INFLUENZA VIRUS OF DIFFERING VIRULENCE FOR BOTH FERRETS AND MAN

C. SWEET, R. A. BIRD, G. L. TOMS,* C. G. WOODWARD AND H. SMITH

From the Department of Microbiology, University of Birmingham, Birmingham B15 2TT

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Summary.—After intranasal inoculation of ferrets with influenza virus the upper respiratory tract infection diminishes during the second day and the onset of this reduction occurs earlier for an attenuated clone (64d) than for a virulent clone (7a) of the recombinant virus A/PR/8/34—A/England/939/69 (H_3N_2). The relevance of pyrexia and the nasal inflammatory response to this reduction in infection has been investigated.

Egg-grown Clone 64d was more thermobile than Clone 7a at normal and pyrexial temperatures when suspended in egg allantoic fluid or phosphate-buffered saline. However, in infected nasal washes, both clones were rapidly inactivated when the washes were incubated at these temperatures.

***In vitro* tests showed that both clones adsorbed to the phagocytes of peritoneal exudates from uninfected ferrets and nasal inflammatory exudates of ferrets infected with both clones. About 90% of the virus was adsorbed after 30 min at 0° or 37° and only 2–14% of this was recovered after treatment with receptor-destroying enzyme followed by freeze-thawing the cells. In contrast, high recoveries (36–112% of that adsorbed) were obtained from red blood cells that were treated similarly. Significant differences were not detected between the clones in either adsorption by or recovery from phagocytes of the different types of exudates.**

Thus pyrexia and the nasal inflammatory cells probably play a major role in the reduction of nasal tract infection but, while pyrexia may have had some influence, no evidence was obtained to indicate that the cells contributed to the earlier reduction of Clone 64d.

PREVIOUS studies on the pathogenesis of influenza virus in ferrets have demonstrated that two clones (7a and 64d) of a recombinant influenza virus A/PR/8/34—A/England/939/69 (H_3N_2) exhibited characteristics of virulence similar to those in man (7a, virulent; 64d, attenuated) (Toms *et al.*, 1976, 1977). Both recombinants replicated to similar titres in ferret nasal mucosa over the first 17–21 h after intranasal inoculation. Thereafter, titres of Clone 64d in nasal washes levelled out at about 10^5 50% egg-bit infectious doses ($EBID_{50}$) per ml and began to fall 29–33 h after infection while titres of Clone 7a continued to rise, reaching a later plateau of 10^6 $EBID_{50}$/ml at 29 h. After a short plateau phase (about 8 h), titres of Clone 7a also declined (Toms *et al.*, 1977). Thus, the decline of titres of Clone 64d in the nasal washes occurred 4–8 h before that of Clone 7a. Once the decline had begun it proceeded equally rapidly for both clones but because of the higher peak titres Clone 7a persisted for longer. Fall in virus titres was preceded by pyrexia, more severe and prolonged for Clone 7a, and a nasal inflammatory response, similar for both clones and marked by a cellular infiltration of 90% polymorphonuclear (PMN) and

* Present address: Department of Virology, University of Newcastle-upon-Tyne.

10% mononuclear (MN) phagocytes. Both febrile and inflammatory responses occurred at about the same time (25–29 h after inoculation) for both clones. Furthermore, both pyrexia and numbers of inflammatory cells were found to correlate significantly with subsequent falls in virus titres in nasal washes and whatever the mechanism of the association between these parameters it occurred sooner for Clone 64d than for Clone 7a (Toms *et al.*, 1977).

Both pyrexia (Fenner *et al.*, 1974) and phagocytes (Mims, 1972; Smith, 1972) are potential defence mechanisms against virus disease. Furthermore, influenza viruses are thermolabile (Hoyle, 1968) and interact with phagocytes both *in vivo* and *in vitro*. Rodent and human PMN and MN phagocytes have adsorbed, ingested and destroyed influenza viruses, sometimes with some damage to the phagocytes, at least as regards their ability to ingest bacteria (Ginsberg and Blackmon, 1956; Boand, Kempf and Hanson, 1957; Hanson, Kempf and Boand, 1957; Inglot and Davenport, 1962; Sawyer, 1969; Alexandrescu *et al.*, 1974; Polezhaev, Makariev and Aleksandrova, 1974; Hackemann, Denman and Tyrrell, 1974; Kolot *et al.*, 1976; Larson and Blades, 1976; Schlesinger, Ernst and Weinstein, 1976). The pattern of phagocyte–virus interaction seems to depend upon the type of phagocyte, the animal species and virus strain (Sawyer, 1969). Perhaps the study most relevant to our work with ferrets was that of Hanson *et al.* (1957), who showed that virus titres in the peritoneal cavities of mice declined with the onset of the inflammatory response due to adsorption of virus on to cells, followed later by phagocytosis. Hence it is likely that pyrexia and the phagocytes of the nasal inflammatory response would have an influence on the respiratory infection in ferrets and experiments *in vitro* related to this hypothesis are described in this paper. As far as we are aware, interactions between ferret phagocytes and influenza virus *in vitro* have not been described previously nor comparisons made of such interactions with strains of differing virulence.

MATERIALS AND METHODS

Virus clones, infectivity assays, haemagglutination (HA) and haemagglutination-inhibition (HI) tests.—These were described by Toms *et al.* (1976).

Inoculation of ferrets and collection of nasal washes.—These were as described by Toms *et al.* (1976, 1977) using phosphate-buffered saline (PBS(A)) for the washes which were used fresh as described below. Samples were kept at −70° for infectivity titrations.

Thermostability of clones.—The stability of Clones 7a and 64d were compared over the range 35–41° because, during infection, although rectal temperatures rise from 38° to 41° (Toms *et al.*, 1976, 1977), nasal temperatures are probably slightly lower.

Egg-grown virus suspensions (64d, $10^{8\cdot5}$ $EBID_{50}$/ml; 7a, $10^{8\cdot0}$ $EBID_{50}$/ml) were diluted (100–300-fold) either in egg allantoic fluid or in PBS(A). Nasal washes were collected from ferrets 25 h after inoculation of 10^6 $EBID_{50}$ of Clones 7a or 64d, when virus contents were similar and near maximum (Toms *et al.*, 1977), and the phagocytes removed by centrifugation (see below).

Initial samples were taken and 3 replicate suspensions incubated at 35, 37, 39 or 41° in water-baths. At hourly intervals up to 8 h samples (0·1 ml) were taken, diluted in 0·9 ml of 1% (w/v) bovine serum albumin (BSA; Armour Pharmaceutical Co. Ltd., Fraction V) and frozen at −70° for subsequent infectivity titrations.

Peritoneal exudate cells from uninfected ferrets.—Ferrets were inoculated i.p. with 30 ml of 0·1% glycogen (oyster glycogen; BDH Ltd.) in PBS(A). Twenty h later approximately 120 ml of PBS(A) were injected i.p. and the fluid allowed to drain out through a 15G × 2 inch B–D Special Needle (Becton, Dickinson and Company, U.K. Ltd.). Cells were collected from the wash by centrifugation at 300 *g* for 10 min at 4° in siliconized tubes and washed once with 20 ml of HBG (Hanks' balanced salt solution with 0·2% BSA and 0·2% glucose). Total and differential cell counts (Toms *et al.*, 1977) were made on a concentrated suspension in HBG. The white blood cells, which contained approximately 90% PMN and 10% MN phagocytes, were then diluted to contain 10^7 total white cells in 0·1 ml of HBG. The cell suspensions also contained contaminating red blood cells and the proportions of these in relation to the white blood cells were determined.

Nasal exudate cells from ferrets infected with Clones 7a and 64d.—Forty-eight h after infection

(when virus contents had been reduced; Toms *et al.*, 1976, 1977) nasal washings were collected from 2 groups of 8 ferrets infected with either Clone 7a or 64d. The nasal tract of each animal was washed 4 times at ½-hourly intervals. The washings were collected in siliconized glassware and centrifuged at 300 *g* for 10 min at 4°. After removal of the supernatant solution the cells were washed once with 20 ml of HBG, the washings discarded, and total and differential counts (Toms *et al.*, 1977) performed. The cells were suspended in HBG at a concentration of 10^7 in 0·1 ml and the residual infectious virus in this suspension was determined.

Nasal and peritoneal cell–virus interaction.—0·1 ml of cell suspension was mixed with 0·1 ml of virus suspension in siliconized glass tubes and held either in ice (0°) or at 37° for 30 min. HBG (0·8 ml) was added, the contents mixed and, after centrifugation (300 *g*, 10 min, 4°), the supernatant solution was removed for infectivity assay of unadsorbed virus. The cell deposit was washed twice in HBG (1·5 ml), resuspended in 1 ml of PBS(A) containing 25% (v/v) RDE (Receptor Destroying Enzyme from *Vibrio cholerae*; Wellcome Reagents Ltd., Beckenham) and 0·2% BSA and incubated at 37° for 60 min. Adsorbed but non-phagocytosed virus should be liberated by this treatment. After centrifugation (300 *g*, 10 min, 4°) the supernatant was removed and titrated for infectivity. Finally, the deposited cells were washed once in HBG (1·5 ml) before resuspending in 1 ml of HBG and freeze-thawing rapidly 3 times in liquid nitrogen to release infectious virus from within the cells. Suitable controls were included to determine the amount of viral inactivation by the procedures employed. Controls for the adsorption process consisted of 0·1 ml of virus suspension and 0·1 ml of HBG (*i.e.* with no cells) incubated at 37° for 30 min with the addition of 0·8 ml of HBG before infectivity titrations. For controls on the elution process 0·1 ml of original virus suspension and 0·9 ml of 25% (v/v) RDE with 0·2% BSA were incubated at 37° for 60 min before titration to determine the inactivation of virus by RDE. Finally, the freeze-thaw procedure was controlled by incubating 0·1 ml of original virus suspension and 0·9 ml of HBG at 37° for 60 min and the suspension frozen and thawed 3 times before infectivity assay to determine the degree of inactivation. These three procedures caused reductions of 0·05–0·15, 0·1–0·4 and 0·1–0·4 $\log_{10}$EBID$_{50}$ in titres respectively for both Clones 7a and 64d in relation to the titres of virus suspensions kept at 0°. The three controls were conducted in each experiment and the appropriate corrections have been made in each table. To check on the validity of using RDE to liberate adsorbed virus and to assess the influence of the presence of contaminating red blood cells in peritoneal exudate cells, experiments were conducted with ferret red blood cells (alone or in the presence of peritoneal exudate cells) in an identical manner to those described above for peritoneal exudate cells.

RESULTS

Thermal stability of Clones 7a and 64d

In egg allantoic fluid the egg-grown clones were inactivated relatively slowly, Clone 64d being slightly more labile than Clone 7a at all the temperatures of incubation and both clones showing a similar increased lability at 39° and 41° compared with 35° and 37° (Table I, Fig. a). In contrast, both egg-grown clones were rapidly inactivated when suspended in PBS(A) but again Clone 64d was more labile than Clone 7a and showed a much greater increase in lability at 39° and 41° compared with 35° and 37° (Table II, Fig. b). In the nasal washes from infected ferrets, which were made with PBS(A) but contained, in addition to virus, materials but not cells washed from the nasal mucosa, both clones were inactivated more rapidly than in PBS(A) alone (Table III) with Clone 64d showing evidence of a greater resistance to destruction than Clone 7a.

Virus interaction with peritoneal cells from uninfected ferrets

The differences in virus infectivity of virus suspensions before and after interaction with peritoneal cells (Table IV) were, in most cases, statistically significant ($P<0·05$) and indicated that 84–98% of virus could have been adsorbed by the cells under the experimental conditions. No differences in behaviour were observed between Clones 7a and 64d at either 0° or 37°. When the cells were treated with RDE little or no virus was released and only small quantities were found in the freeze-thawed extracts of the cells. There were no differences between the clones and total recoveries from the cells (4–11%) were poor.

The preparations of peritoneal PMN and MN phagocytes were contaminated to

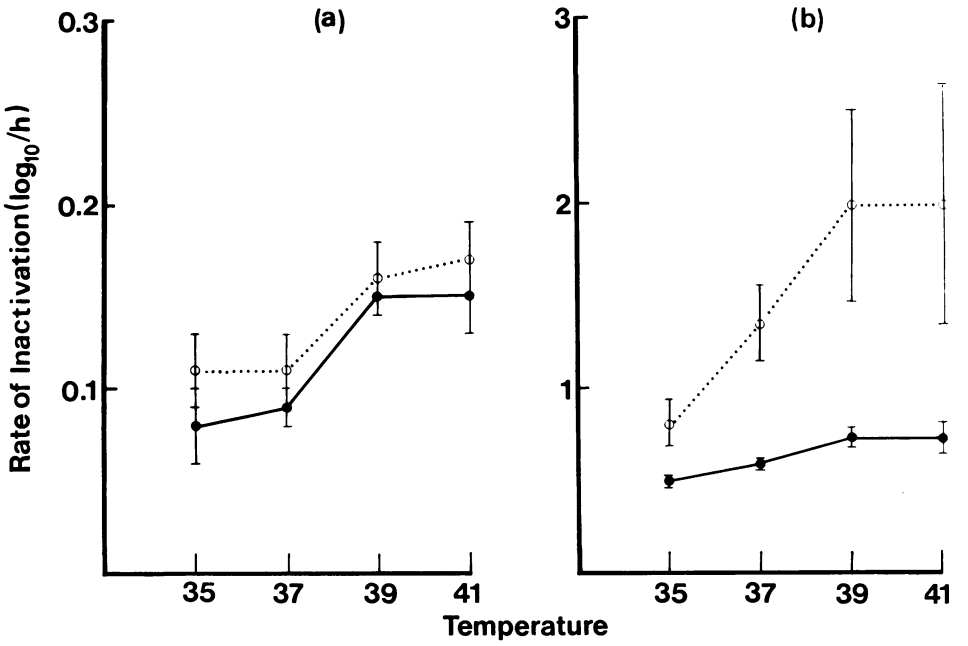

FIG.—The rate of inactivation (log_{10}/h) of Clones 7a (●—●) and 64d (○. . . ○) diluted in allantoic fluid (a) or PBS(A) (b). The inactivation rates were determined from linear regression analysis of the data in tables I and II; the bars on each point represent the s.e. mean.

TABLE I.—*Thermal Stability of Egg-grown Clones 7a and 64d Suspended in Egg Allantoic Fluid*

Temperature of incubation	Infectivity (log_{10}EBID$_{50}$/ml) of suspension containing: 7a					64d				
	Initially	After 2 h	After 4 h	After 6 h	After 8 h	Initially	After 2 h	After 4 h	After 6 h	After 8 h
41°	5·8(0·2)	5·5(0·1)	4·8(0·1)	4·8(0·1)	4·6(0·2)	5·7(0·3)	5·1(0·1)	4·6(0·1)	4·4(0·1)	4·0(0·1)
39°	5·8(0·2)	5·3(0·1)	5·2(0·1)	4·7(0)	4·7(0)	5·7(0·3)	5·0(0·2)	4·7(0·1)	4·3(0·2)	4·5(0·1)
37°	5·8(0·2)	5·5(0·3)	5·2(0·1)	5·1(0·1)	4·9(0·1)	5·7(0·3)	5·2(0·1)	4·9(0·1)	5·0(0·2)	4·5(0·1)
35°	5·8(0·2)	5·5(0·1)	5·5(0·2)	5·4(0·1)	5·2(0·2)	5·7(0·3)	5·4(0)	5·0(0)	5·0(0·2)	4·7(0·1)

The figures are from a representative experiment and are the means (s.e. mean in parentheses) of samples taken from 3 replicate incubated aliquots. Additional figures obtained at 1, 3, 5 and 7 h are not shown.

TABLE II.—*Thermal Stability of Egg-grown Clones 7a and 64d Suspended in PBS(A)*

Temperature of incubation	Infectivity (log_{10}EBID$_{50}$/ml) of suspension containing: 7a					64d				
	Initially	After 2 h	After 4 h	After 6 h	After 8 h	Initially	After 2 h	After 4 h	After 6 h	After 8 h
41°	5·7(0·1)	3·7(0·5)	2·0(0·5)	<1·5	<1·5	5·5(0)	<1·5	<1·5	<1·5	<1·5
39°	5·7(0·1)	4·3(0·2)	2·4(0·3)	<1·5	<1·5	5·5(0)	<1·5	<1·5	<1·5	<1·5
37°	5·7(0·1)	4·9(0·1)	3·0(0·3)	1·8(0·2)	<1·5	5·5(0)	2·1(0·3)	<1·5	<1·5	<1·5
35°	5·7(0·1)	5·0(0·1)	4·1(0·3)	2·9(0·5)	2·2(0·6)	5·5(0)	3·1(0·5)	1·6(0)	<1·5	<1·5

The figures are from a representative experiment and are the means (s.e. mean in parentheses) of samples taken from 3 replicate incubated aliquots. Additional figures obtained at 1, 3, 5 and 7 h are not shown.

TABLE III.—*Stability of Clones 7a and 64d in Nasal Washes from Infected Ferrets*

Temperature of incubation	Infectivity ($log_{10}EBID_{50}$/ml) of wash* containing: 7a Initially	7a After 1 h	7a After 2 h	7a After 3 h	64d Initially	64d After 1 h	64d After 2 h	64d After 3 h
41°	4·7(0·1)	<1·5	<1·5	<1·5	4·6(0·1)	1·6(0·1)	<1·5	<1·5
39°	4·7(0·1)	<1·5	<1·5	<1·5	4·6(0·1)	1·7(0·1)	<1·5	<1·5
37°	4·7(0·1)	1·6(0·1)	<1·5	<1·5	4·6(0·1)	2·0(0·3)	<1·5	<1·5
35°	4·7(0·1)	<1·5	<1·5	<1·5	4·6(0·1)	2·5(0·2)	1·5(0)	<1·5

* The washes were made with PBS(A).

The figures are from a representative experiment and are the means (s.e. mean in parentheses) of samples taken from 3 replicate incubated aliquots.

TABLE IV.—*The Interactions of Clones 7a and 64d with Peritoneal Cells from Uninfected Ferrets*

Clone	Temperature of adsorption	No. of white blood cells	Infectivity ($log_{10}EBID_{50}$): Added	Unadsorbed	Adsorbed (% added)	Eluted* by RDE	In cell† extract	Total recovery (% adsorbed)
7a	0°	10^7 (1 : 1)‡	6·87(0·1)	6·04(03)	6·80 (85)	5·63(0·1)	5·43(0·2)	5·80 (10)
	37°		6·80(0·2)	5·28(0·4)§	6·79 (98)	5·49(0·1)	5·01(0·1)	5·60 (7)
64d	0°		6·86(0·1)	5·92(0·1)§	6·81 (89)	5·76(0·1)	5·41(0·1)	5·86 (11)
	37°		6·77(0·1)	5·80(0·2)§	6·72 (89)	5·44(0·1)	5·00(0·2)	5·57 (7)
7a	0°	10^7 (4 : 1)	7·27(0·1)	6·07(0·1)§	7·24 (91)	6·08(0·1)	5·83(0·1)	6·27 (11)
	37°		7·20(0·4)	6·42(0·2)	7·12 (84)	5·56(0·2)	5·36(0·2)	5·77 (5)
64d	0°		7·23(0·3)	6·00(0·1)§	7·2 (94)	5·70(0·1)	5·46(0·2)	5·90 (5)
	37°		7·07(0·1)	5·93(0)§	7·03 (91)	5·51(0·5)	5·00(0·1)	5·60 (4)
7a	0°	10^7 (10 : 1)	7·27(0·1)	6·08(0·2)§	7·24 (91)	5·62(0·5)	6·19(0·2)	6·29 (11)
64d	0°		7·23(0·3)	5·73(0·1)§	7·22 (98)	5·72(0·3)	5·66(0·3)	5·99 (6)

* In PBS(A)+BSA at 37° for 60 min.

† Cells frozen and thawed 3 times.

‡ Ratio of white blood cells : contaminating red blood cells.

§ These figures were significantly ($P<0·05$) different from those of the added virus.

The results are from 3 typical experiments and are the means (s.e. mean in parentheses) of samples taken from 3 replicate incubated aliquots. The figures for added virus are those obtained on suspensions incubated for 30 mins at 0° or 37° without cells. Other figures are corrected for any inactivation of virus that occurred on incubation with RDE in PBS(A)+BSA for 60 min at 37° and during freeze-thawing 3 times.

different degrees with red blood cells but this did not appear to influence either adsorption or elution of virus in experiments described in Table IV. The first experiment in Table V shows that, when a badly contaminated preparation of peritoneal exudate cells was diluted to contain 5×10^5 white blood cells in the presence of 10^7 red blood cells, 89–93% of Clone 7a was adsorbed but 89–112% was recovered, predominantly by treatment with RDE, although some was present in the frozen and thawed cell extract. Similarly (Second experiment, Table V), 10^7 red cells from ferret blood adsorbed 89% of Clone 7a and 71% could be recovered; with 10^6 red

TABLE V.—*The Interaction of Clones 7a and 64d with Red Blood Cells from Uninfected Ferrets*

Clone	Temperature of adsorption	No. of red blood cells	Infectivity ($log_{10}EBID_{50}$): Added	Unadsorbed	Adsorbed (% added)	Eluted* by RDE	In Cell† extract	Total recovery (% adsorbed)
7a	0°	10^7‡	6·87(0·1)	5·87(0·4)	6·82 (89)	6·77(0·1)	6·18(0·1)	6·87 (112)
7a	37°	10^7‡	6·87(0·1)	5·73(0·1)§	6·84 (93)	6·66(0·1)	6·20(0·2)	6·79 (89)
7a	0°	10^7	7·27(0)	5·51(0·1)§	7·22 (89)	7·07(0)	Not done	7·07 (71)
7a	0°	10^6	7·27(0)	6·49(0·1)§	7·19 (83)	6·74(0·3)	Not done	6·74 (36)
7a	0°	10^7	7·67(0·1)	6·57(0·2)§	7·63 (91)	7·00(0·1)	6·90(0·3)	7·25 (42)
64d	0°	10^7	7·40(0·2)	6·52(0·1)§	7·34 (83)	7·10(0·1)	6·40(0·1)	7·18 (69)

* In PBS(A)+BSA at 37° for 60 min.
† Cells frozen and thawed 3 times.
‡ Sample also contained 5×10^5 white blood cells.
§ These figures were significantly ($P<0·05$) different from those of added virus.
The results are from typical experiments and are the means (s.e. mean in parentheses) of samples taken from 3 replicate incubated aliquots. The figures for added virus are those obtained on suspensions incubated for 30 min at 0° or 37° without cells. Other figures are corrected for any inactivation of virus that occurred on incubation with RDE in PBS(A)+BSA for 60 min at 37° and during freeze-thawing 3 times.

TABLE VI.—*The Interactions of Clones 7a and 64d with Nasal Cells from Infected Ferrets*

Clone	Temperature of adsorption	No. of cells (source)*	Infectivity ($log_{10}EBID_{50}$): Added	Unadsorbed	Adsorbed (% added)	Eluted† by RDE	In cell‡ extract	Total recovery (% adsorbed)
7a	0°	10^7 (7a)	7·67(0·1)	7·10(0·1)§	7·53 (72)	5·93(0·4)	6·24(0·2)	6·41 (8)
7a	37°	10^7 (7a)	7·60(0·2)	6·35(0·3)§	7·58 (96)	6·12(0)	5·92(0·2)	6·33 (6)
64d	0°	10^7 (7a)	7·40(0·2)	6·66(0·2)	7·31 (82)	6·30(0·2)	5·94(0·2)	6·46 (14)
64d	37°	10^7 (7a)	7·53(0·3)	6·47(0·3)	7·50 (93)	5·43(0·4)	5·60(0·2)	5·82 (2)
7a	0°	10^7 (64d)	7·67(0·1)	6·17(0·4)§	7·66 (97)	6·23(0·2)	6·08(0·1)	6·46 (6)
7a	37°	10^7 (64d)	7·60(0·2)	6·43(0·3)§	7·57 (93)	5·57(0·2)	6·36(0·7)	6·42 (7)
64d	0°	10^7 (64d)	7·40(0·2)	6·18(0·2)§	7·37 (94)	6·26(0·1)	5·91(0·5)	6·42 (11)
64d	37°	10^7 (64d)	7·53(0·3)	6·73(0·1)	7·45 (84)	5·43(0·3)	5·65(0·1)	5·86 (2)

* The cells came from either Clone 7a infected animals (and contained $10^{4·8}EBID_{50}$ of virus with 10^7 cells) or Clone 64d infected animals (and contained $10^{2·7}$ $EBID_{50}$ of virus with 10^7 cells).
† In PBS(A)+BSA at 37° for 60 min.
‡ Cells frozen and thawed 3 times.
§ These figures were significantly ($P<0·05$) different from those of added virus.
The results are from a typical experiment and are the means (s.e. mean in parentheses) of samples taken from 3 replicate incubated aliquots. The figures for added virus are those obtained on suspensions incubated for 30 min at 0° or 37° without cells. Other figures are corrected for any inactivation of virus that occurred on incubation with RDE in PBS(A)+BSA for 60 min at 37° and during freeze-thawing 3 times.

blood cells (Third experiment, Table V) adsorption and recovery were 83 and 36% respectively. In a final experiment (Table V) with Clone 64d, as with Clone 7a, similar figures for adsorption and elution were observed. Thus, if the adsorption by the peritoneal exudate cells had been primarily due to the contaminating red blood cells, the subsequent recoveries of virus would have been much larger.

Virus interaction with nasal inflammatory cells from ferrets infected with Clones 7a and 64d

Yields of cells (about 5×10^4/10 ml nasal wash) from uninfected ferrets were too low for adequate experimental work and therefore cells (about 90% PMN and 10% MN phagocytes) were collected from infected animals where yields were 100-fold higher due to the inflammatory response (Toms *et al.*, 1977). Red blood cell contamination was not observed. The results of adsorption and elution studies with the nasal cells were similar to those with peritoneal cells. The differences between virus infectivities of suspensions before and after treatment with the cells were statistically significant ($P<0{\cdot}05$) in most cases and indicated 72–97% of virus may have been adsorbed (Table VI). No differences in behaviour were observed between the clones at 0° or 37° on cells from animals infected with either clone. Again recoveries of the clones by treatment with RDE were poor as were those found in frozen and thawed extracts of the cells and no differences between the clones were seen.

DISCUSSION

Previous work (Toms *et al.*, 1977) on nasal infection of ferrets with influenza virus suggested a link between the nasal inflammatory response, the coincident pyrexia and subsequent falls of virus titres in the nose. The decline in virus titres began sooner for the attenuated Clone 64d than for the virulent Clone 7a. A causal relation between the host defences and the reduction in virus titres was therefore possible. The existence of such a relation would receive support by the demonstration of a thermal lability of the two clones at pyrexial temperatures with Clone 64d being less stable than Clone 7a, and/or phagocytosis and inactivation of the two clones by the phagocytes of the nasal inflammatory response with Clone 64d being more prone to inactivation than Clone 7a.

In two media—one containing possibly protective protein and the other a simple buffer—both clones were thermolabile at pyrexial temperatures and Clone 64d was more thermolabile than Clone 7a. Assuming that ferret-grown virus has similar thermal characteristics to egg-grown virus it appears that the pyrexia which accompanies the nasal infection could contribute to the destruction of both clones, Clone 64d succumbing more rapidly than Clone 7a. The temperature lability experiments with ferret-grown virus in the crude nasal wash do not invalidate this conclusion, since the extreme lability of both clones in the nasal wash was probably due to virus inhibitors washed from the nasal tract or liberated from phagocytes before or after collection of the washes (see below).

Both peritoneal exudate cells and nasal inflammatory cells, which consisted of about 90% PMN and 10% MN phagocytes, adsorbed most of the virus from suspensions when *in vitro* tests were conducted as described above. If the concentrations of cells or virus were reduced, a lower proportion of inoculum virus appeared to be adsorbed and at 10–100-fold less concentration of both cells and virus significant adsorption could not be detected (unpublished results). Peritoneal exudate cells were used as well as nasal inflammatory cells because the latter could not be obtained in sufficient quantity from uninfected ferrets. Also, tests with uninfected cells were needed because they might have yielded different results from those with cells from infected ferrets which may have been harmed by prior interaction with virus (Sawyer, 1969;

Larson and Blades, 1976; Schlesinger *et al.*, 1976). In the event, the cells from the two sources behaved similarly in the tests. Only small amounts of virus were recoverable from the phagocytes by treatment with RDE or freeze-thawing, procedures which did recover adsorbed virus from red blood cells. Surprisingly, virus-cell interactions occurred equally well at 0° and 37°. However, the phenomenon has been observed previously in studies with influenza virus and lymphocytes (Hackemann *et al.*, 1974).

Although the disappearance of virus infectivity from suspension on mixing with the phagocytes was probably due to adsorption, direct evidence on this point is lacking because, unlike the large recoveries obtained from red blood cells, little virus infectivity was recoverable from the phagocytes by treatment with RDE and freeze-thawing. The lack of recovered virus could have been due to inactivation of suspension virus without adsorption or to receptors on the phagocytes binding virus externally and, unlike those on red blood cells, the bonds being unbroken by treatment with RDE and freeze-thawing. However, it is more likely that inactivation occurred after adsorption and ingestion of virus by these actively phagocytic cells, as has been reported in other studies with phagocytes of other animal species (see the introduction). Whatever the mechanism, clearly destruction of virus infectivity occurred and the two clones showed no marked differences in behaviour.

Since phagocytes mobilized by the mucosal inflammatory response will be in close proximity to virus released from the epithelial cells, either in the mucosa itself or at its surface, it is likely that the inactivation of virus observed in the *in vitro* tests contributes to the rapid decline of both virus clones in the nasal tract on the second day of infection (Toms *et al.*, 1976, 1977). However, no differences could be detected between virulent or attenuated clones, either in degree of apparent adsorption to peritoneal or nasal cells or in extent of recovery of infectivity from such cells. Furthermore, the nasal cells induced by infection with either clone behaved similarly with both clones in subsequent phagocytosis tests *in vitro*. Thus, no evidence was obtained to suggest that the earlier decline of nasal titres of Clone 64d could be attributed to the activity of the nasal inflammatory cells. Differences between the two clones in interaction with phagocytes could occur and be significant in the different behaviour of the clones *in vivo* but they were not detected in the relatively insensitive tests described here.

Other inhibitory factors which may play a role in control of nasal infection *in vivo* have been detected. The lability studies on the two clones in the crude nasal washes revealed a rapid inactivation or destruction of virus even at normal temperatures. The mucous secretions present in the nasal washes could contain humoral inhibitors which are known to inactivate influenza virus (Smorodintsev, 1960; Davenport, 1961). Nasal washes could also contain viricidal materials liberated either from phagocytes *in vivo* or, as artefacts, during collection of the washes. If such materials were present *in vivo* they might have contributed to the rapid decline of both clones on the second day after infection, but not to the earlier demise of Clone 64d, because this clone appeared to be somewhat more stable to their action than Clone 7a, at least in nasal washes. The role of these possible inhibitors will be the subject of future research.

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