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THE RELATION OF INTERFERON AND NONSPECIFIC INHIBITORS TO VIRUS LEVELS IN NASAL WASHES OF FERRETS INFECTED WITH INFLUENZA VIRUSES OF DIFFERING VIRULENCE

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Summary.—Two clones (7a, virulent; 64d, attenuated) of a recombinant influenza virus (A/PR/8/34-A/ENGLAND/939/69 (H_3N_2)) were inactivated at the same rate by viral inhibitors present in nasal washes taken from both Clone 7a- and Clone 64d-infected ferrets.

Both clones induced similar levels of interferon in the nasal washes of infected animals. The onset and rise of interferon production occurred at the same time for both clones, and was associated with a decline in virus titres. In addition, both clones showed a similar sensitivity to interferon.

Thus, although nonspecific inhibitors and interferon may play a role in reducing nasal tract infection caused by both clones, the differences in virulence of the 2 clones do not appear to be determined by differential induction of, or resistance to, these host defence mechanisms.

PREVIOUS STUDIES with 2 clones of a recombinant influenza virus (A/PR/8/34-A/ENGLAND/939/69 (H_3N_2)) showed that they exhibited differences in virulence for ferrets (Clone 7a, virulent; Clone 64d, attenuated) similar to those found for man (Toms *et al.*, 1976, 1977). In particular, on the basis of virus titres in nasal washes, Clone 7a produced a greater and more persistent upper respiratory tract infection in ferrets than Clone 64d. Reduction of the nasal tract infection occurred for both clones as a result of interaction of virus with phagocytes of the nasal inflammatory response and of the inhibitory effect of pyrexia on virus replication (Toms *et al.*, 1977; Sweet *et al.*, 1977, 1978). The earlier reduction of virus titres of the attenuated Clone 64d appeared to be due not to greater inactivation by phagocytes but, in part at least, to its greater instability and inability to replicate at pyrexial temperatures compared with Clone 7a (Sweet *et al.*, 1977, 1978).

Other nonspecific host factors may also be involved, both in defence against influenza, and in the differential virulence of strains. Different nonspecific inhibitors of influenza viruses designated α, β or γ exist in sera and secretions of many animals including ferrets (Krizanova and Rathova, 1969); they may also be present in nasal washes. Resistance to β-inhibitors in the serum of mice correlated with increased virulence in one study (Briody, Cassel and Medill, 1955) but not in others (Barb and Takatsy, 1972; Hirst, 1947; Raut *et al.*, 1975). In previous work, Sweet *et al.* (1977) examined the thermostabilities of Clones 7a and 64d, and found that both clones were rapidly inactivated at 37° when nasal washes of infected animals were incubated *in vitro*, Clone 64d more so than Clone 7a. However, these nasal washes may have contained materials in addition to the normal inhibitors. Nasal washing had been performed with phosphate-buffered saline (Dulbecco A, PBS(A)), and this may have released virucidal materials such as lysosomal enzymes from the nasal inflammatory phagocytes.

Interferon appeared to be an important

defence mechanism in some studies on influenza in mice (Haller *et al.*, 1979; Suzuki *et al.*, 1975; Zee *et al.*, 1979). Also, during infection with influenza virus, interferon has been demonstrated in the nasal washes of man (Gresser and Dull, 1964; Jao, Wheelock and Jackson, 1965, 1970; Smorodintsev *et al.*, 1971; Hill *et al.*, 1972) and in nasal washes and turbinates of ferrets (Small *et al.*, 1976; Haff *et al.*, 1966). However, its precise role in limiting nasal tract infection was unclear (Small *et al.*, 1976; Haff *et al.*, 1966; Pinto, Haff and Stewart, 1969).

In this paper, the roles of nonspecific inhibitors and interferon in defence against influenza virus in the nasal tract of ferrets are examined together with the possibility that differential induction of, or resistance to, these 2 factors may contribute to the differences in virulence between the 2 virus clones.

MATERIALS AND METHODS

Viruses.—Clones 7a and 64d of the recombinant influenza virus A/PR/8/34-A/ENGLAND/939/69 (H_3N_2) were described by Sweet, Stephen and Smith (1974*b*) together with the preparation of seed and working stocks.

Semliki Forest Virus (SFV 31) for assay of interferon was kindly supplied by Professor D. Burke, Warwick University, England. Stocks were prepared by intracerebral inoculation of 40–50 3–4-day-old mice with *ca* one 50% tissue culture dose (TCD_{50})/mouse (see later). Brains were collected 24–28 h after inoculation, homogenized in 40–50 ml (1 ml/brain) of Earle's Basal Medium (Eagle) (BME) (Gibco Biocult, Paisley, Scotland) supplemented with 7% foetal calf serum (FCS), 2mM glutamine, 0·18% sodium bicarbonate, 100 u/ml penicillin and 100 μg/ml streptomycin, and centrifuged at 12,000 *g* (30 min, 4°). The supernatant, containing *ca* 10^4 TCD_{50}/ml of virus, was dispensed in 2ml aliquots and stored at −70°.

Infectivity assays.—Influenza virus was assayed using the egg-bit technique of Fazekas de St Groth and White (1958) as described previously by Sweet, Stephen and Smith (1974*a*).

Semliki Forest Virus (SFV) was assayed using the dye-uptake method of Finter (1969). Primary ferret kidney cells were grown to confluency in 100mm square Repli dishes (Sterilin) in Hanks' BME supplemented with 10% FCS, 2mM glutamine, 0·035% sodium bicarbonate, 100 u/ml penicillin and 100 μg/ml streptomycin at 37° in 5% CO_2/95% air, followed by a further 72h incubation in Earle's BME supplemented as described above but with 10% FCS. The monolayers were then inoculated with appropriate 10-fold dilutions of the virus sample (2 ml/well) and incubated for 24 h at 35°. Neutral red solution was added to the monolayers to give a final concentration of 4×10^{-5} g/ml of medium, and incubated for a further 2 h at 35°. The monolayers were washed twice with PBS(A) and the stain retained by the viable cells was eluted with 3 ml of 1:1 ethanol:0·1M sodium citrate buffer (pH 4·2). The absorbance of the sample was read at 540 nm using a Unicam SP1800 spectrophotometer, and plotted (as percentage of the absorbance obtained from stained cells of control monolayers not treated with virus) against virus dilutions. The dilution of virus required to reduce the absorbance to 50% of the control value was considered to contain one TCD_{50}.

Inactivation of Clones 7a and 64d by nonspecific inhibitors in nasal washes.—The nasal tracts of ferrets infected with 10^6 50% egg-bit infectious doses ($EBID_{50}$) of Clone 7a or 64d, were washed 24 h and 25 h after infection as described previously (Toms *et al.*, 1976) but using Eagle's Minimum Essential Medium (MEM) supplemented with 0·18% sodium bicarbonate, 100 u/ml penicillin and 100 μg/ml streptomycin, instead of PBS(A), to minimize disruption of phagocytes. Washes from the respective animal were pooled and centrifuged (300 *g*, 10 min, 4°) to remove phagocytes and epithelial cells. Initial samples were taken and 3 replicate suspensions (virus contents approximately $10^{5.0}$ and $10^{4.0}$ $\log_{10}$ $EBID_{50}$/ml for Clone 7a and 64d respectively) were incubated in a water bath at 37°. Samples were withdrawn at regular intervals up to 6 h and frozen with 1% (w/v) Bovine Serum Albumin (Armour Pharmaceutical Co., Eastbourne, England) at −70° for subsequent infectivity titrations. Controls with which egg-grown virus was diluted in either nasal washes from non-immune, non-infected animals or Eagle's MEM, were also monitored for inactivation at 37°. The inactivation rates ($\log_{10}$ $EBID_{50}$/h) for experimental and control samples were calculated from plots of mean virus contents of the 3 replicate suspensions against time over 6 h; these plots were essentially linear.

Preparation of putative interferon from nasal washes.—The nasal tract of ferrets infected with either Clone 7a or 64d as described above were washed with 7 ml of Earle's BME, supplemented as above but with 1% FCS, at regular intervals up to 72 h after inoculation. Nasal washes (5 ml) were centrifuged (300 *g*, 10 min, 4°) and dialysed against 0·9% NaCl/HCl (pH 2) for 48 h, neutralized by dialysis against PBS(A) (pH 7·3) for 24 h, and stored at −70°.

Assay of putative interferon in nasal washes.—

Primary ferret kidney cells, grown to confluency as described above, were treated for 18 h with a 1 in 10 dilution of the treated nasal wash, and then washed twice with 1 ml of Earle's BME supplemented as above but with 1% FCS to remove any nonspecific inhibitors that might have been present before infecting with *ca* 200 TCD_{50} of SFV. The amount of interferon present in the nasal washes was indicated by the percentage of cells surviving after 24 h as determined by the dye-uptake method described above. The absorbance obtained from cell monolayers treated with nasal wash only was considered equivalent to 100% survival of cells and the absorbance obtained from monolayers treated with SFV only was considered equivalent to 0% survival. The numerous samples from the experiments described in Results could not be compared in the same assay. To correct for any variation in interferon sensitivity using different batches of ferret kidney cells, one sample (from a nasal washing taken 46 h after inoculation with Clone 7a) was kept as an internal standard in all assays. The correction factor for different assays using this standard varied from 0·9 to 1·3.

Determination of the sensitivity of the 2 clones to putative interferon.—Monolayers of ferret kidney cells, grown to confluency as described above, were treated for 18 h with a 1 in 10 dilution of a pool of nasal washes prepared for interferon assay and in which assays using SFV had indicated relatively high interferon levels. The monolayers were washed twice as described in the previous section, and inoculated with 1·5 ml of either Clone 7a ($10^{2.8}$ or $10^{3.8}$ $EBID_{50}$/ml) or Clone 64d ($10^{3.5}$ or $10^{4.5}$ $EBID_{50}$/ml). After incubation at 35° for 24 h, the yields of virus from interferon-treated and non-treated cells were determined using the egg-bit technique.

McCoy cells.—These were obtained from Flow Laboratories (Scotland) and grown on Eagle's MEM supplemented with 5% FCS, 2mM glutamine, 0·18% sodium bicarbonate, 100 u/ml penicillin and 100 μg/ml streptomycin.

This cell line has been found to be indistinguishable in karyotype and antigenic constitution from the mouse L929 tumour cell line (Gordon *et al.*, 1972), and was used in some comparative experiments with the putative interferon preparations as described in Results.

RESULTS

Inactivation of Clones 7a and 64d by nonspecific inhibitors in nasal washes

Results in Table I show that *in vitro* the rates of inactivation of both clones in the nasal washes of infected animals at 37° were greater than in washes from uninfected animals or in the medium used for nasal washing (Eagle's MEM). This suggests that viral inhibitors, acting directly on the virus clones, were present in these washes. However, the rates of inactivation of both clones were similar in all media (Table I).

TABLE I.—*Inactivation of Clones 7a and 64d in nasal washes from infected and uninfected ferrets and the wash medium at 37°*

Virus origin	Rate of inactivation ($log_{10}EBID_{50}$/h) of clones 7a	64d
Nasal wash* of infected animals	0·42 (0·06)	0·44 (0·09)
Egg-grown virus suspended in nasal wash* of uninfected animals	0·21 (0·02)	0·19 (0·03)
Egg-grown virus suspended in Eagle's MEM	0·26 (0·05)	0·27 (0·04)

Rates of inactivation were obtained from linear plots of virus titres at intervals up to 6 h. The figures (s.e. in parentheses) are the means of 3–5 experiments.

* The nasal washes were performed with Eagle's MEM; the approximate virus concentrations were $10^{5.0}$ and $10^{4.0}$ log_{10} $EBID_{50}$ for Clones 7a and 64d respectively.

Evidence for production of interferon by Clones 7a and 64d in the nasal tract

Pretreated nasal wash preparations (especially those taken between 34 h and 53 h after inoculation) protected ferret kidney cells against the challenge virus SFV (see Fig. b) in an assay in which ferret kidney-cell monolayers were washed thoroughly after treatment with the nasal wash preparation to remove nonspecific inhibitors that might be present before the challenge virus inoculum was added. Thus the protective material in the preparations was acid-stable, non-dialysable and inhibited a virus unrelated to that which induced it, all of which are properties of interferon (Lockart, 1973). Additional evidence that this activity was due to interferon is as follows. Firstly, in 3 experiments the nasal wash preparation showed species specificity; while these protected ferret kidney cells against SFV, they did

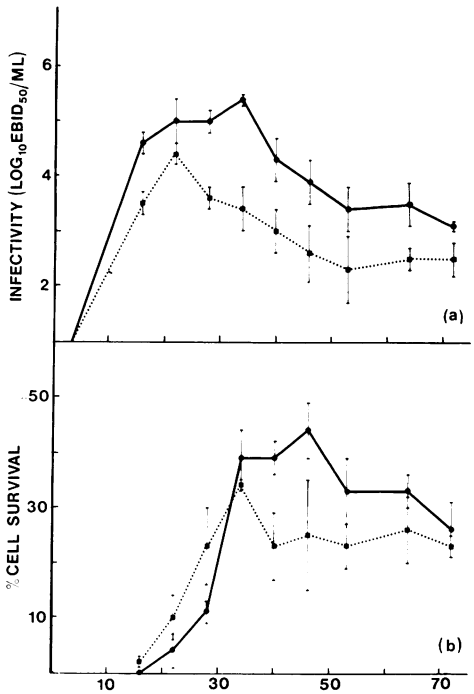

FIG.—Mean viral titres (a) and mean interferon levels (b) in the nasal washes of animals inoculated intranasally with Clone 7a (●——●) or Clone 64d (■ - - - ■). Interferon levels are expressed as percentage of ferret kidney cells surviving after treatment with 1 in 10 dilution of the nasal wash preparation and challenge with SFV, compared with control. The bars represent the s.e. of results involving 3 animals for each clone.

not protect McCoy cells against infection with the virus (the tests were similar to those described with ferret kidney cells in Materials and Methods, and in these tests mouse interferon kindly supplied by Professor D. Burke protected the McCoy cells from the infection with SFV). In one experiment, for example, whereas 2 preparations from ferrets infected with Clones 7a and 64d protected 45% and 36% of the ferret kidney cells respectively, neither protected McCoy cells. Secondly, after treatment of cells with 2 similar preparations, there was no significant reduction of yield of either Clone 7a or 64d when they were grown in McCoy cells for 24 h, while yields were substantially reduced in ferret cells. For example, the yields of Clone 7a after infecting McCoy cells as described for ferret cells in Materials and Methods and incubating for 24 h at 35° were not significantly different for untreated cells and those treated with 2 preparations from nasal washes (treated 0·3 and 0·2 $\log_{10}$ EBID$_{50}$ lower than untreated), whereas significant differences were recorded from similar experiments with ferret kidney cells (treated 1·7 and 1·1 $\log_{10}$ EBID$_{50}$ lower than untreated cells). Thirdly, 2 nasal wash preparations that respectively protected 32% and 49% of ferret cells against SFV infection produced no significant *direct* inactivation of either Clone 7a or 64d; the rates of inactivation of Clone 7a mixed with a 1 in 10 dilution of the 2 preparations made in Earle's BME supplemented with 1% FCS were −0·10 and −0·10 log/h compared with −0·08 log/h for the control, while rates of inactivation of 64d were −0·09 and −0·07 log/h compared with −0·07 log/h for the control. Finally, centrifugation at 100,000 *g* for 2 h did not appear to affect the levels of activity in nasal wash preparations; thus 1 in 10 dilutions of these centrifuged preparations from nasal washes taken at 53 h after inoculation protected 32%, 17% and 62% of the ferret kidney cells, while similar dilutions of 3 uncentrifuged preparations from washes taken at the same hour after inoculation from 3 other animals protected 32%, 22% and 47% of the cells.

Thus it was reasonable to assume that the material in the treated nasal washes that protected cells from SFV and influenza virus infection was interferon. However, when the nasal wash preparations were added to the ferret kidney cells at the same time as the SFV (instead of 18 h before) protection was noted although it was often less, but never more, than the level of protection observed when the preparations were added 18 h before the addition of SFV. Ferret interferon might be acting rapidly, as has been described for human and mouse interferons (Dianzani and Baron, 1975, 1977), or SFV might

TABLE II.—*Sensitivity of Clones 7a and 64d to ferret interferon*

Test virus (Expt No.)	Inoculum (log $EBID_{50}$/ml)	Virus yields* in ferret kidney cells infected: Without interferon Yield	With interferon from 7a infected animals Yield	7a infected animals % Reduction	With interferon from 64d infected animals Yield	64d infected animals % Reduction
7a (1)	2·8	4·3 (0·2)†	2·4 (0·1)	99	NT	—
	3·8	5·0 (0·1)	3·2 (0·2)	98	NT	—
7a (2)	2·8	3·6 (0·3)	2·4 (0·1)	94	2·3 (0·1)	95
	3·8	4·4 (0·3)	3·2 (0·2)	90	3·4 (0·1)	94
7a (3)	2·8	3·0 (0·4)	1·4 (0·3)	> 99	1·8 (0·1)	> 99
	3·8	3·7 (0·1)	2·6 (0·1)	92	2·8 (0·1)	87
64d (1)	3·5	3·8 (0·0)	2·7 (0·2)	92	NT	—
	4·5	4·9 (0·2)	4·1 (0·1)	84	NT	—
64d (2)	3·5	3·8 (0·2)	2·8 (0·1)	90	2·7 (0·1)	92
	4·5	4·7 (0·1)	4·0 (0·2)	81	3·7 (0·2)	90
64d (3)	3·5	3·1 (0·1)	1·8 (0·2)	> 99	2·4 (0·2)	98
	4·5	4·1 (0·3)	3·7 (0·3)	60	3·6 (0·3)	68

NT = Not tested.
* Log_{10} $EBID_{50}$/ml after 24 h at 35°.
† Figures in parentheses are the standard errors.

act slowly on ferret kidney cells allowing the interferon to render protection.

The relation of interferon production with virus titres in nasal washes

The Figure shows the mean levels of virus and interferon in nasal washes taken at intervals up to 72 h after intranasal inoculation of 3 animals respectively with either Clone 7a or 64d. As noted previously (Toms *et al.*, 1977), Clone 7a produced a greater and more persistent upper respiratory tract infection than Clone 64d. However, this was associated with high mean levels of interferon (Fig. b). Indeed, examination of the results for individual animals showed that the greater the virus titres, the higher the interferon levels, and this occurred for infections with both clones. Interferon levels began to rise between 22 and 28 h after infection for both clones when virus titres were at or near their peak. Maximal levels were reached at 34 h for Clone 64d, when virus titres were declining, but continued to rise for Clone 7a until 40–46 h, when Clone 7a virus titres also began to decline (Fig.). Thus there appeared to be a temporal relation between the rise in interferon levels and the decline of virus titres.

Sensitivity of Clones 7a and 64d to the interferon in nasal washes of infected ferrets

The effect of interferon on reducing virus yields of Clones 7a or 64d in ferret kidney cells is shown in Table II. The yields of both clones in all 3 experiments were considerably reduced in interferon-treated cells compared to untreated cells irrespective of whether the interferon was induced in Clone 7a- or 64d-infected animals. Although there was a tendency for the yields of Clone 7a to be reduced more than those of Clone 64d, these differences were not significant ($P > 0·05$). The reduction in virus yields in the cells treated with the preparations from the nasal washes could not have been due to nonspecific inhibitors for the reasons stated earlier.

DISCUSSION

Nasal washings, performed with Eagle's MEM, inactivated Clones 7a and 64d but did so at a much slower rate than those performed with PBS(A) in previous work (Sweet *et al.*, 1977). However, the inactivation rates for both clones were higher than those in nasal washes of uninfected animals or in the wash medium, suggesting that viral inhibitors, acting

directly on both clones, are induced in the nasal tracts of ferrets as early as 24 h after infection. Whether these inhibitors are of the α-, β- or γ-type found in ferret sera (Krizanova and Rathova, 1969) is unknown, but they may play a role in reducing the nasal tract infection, possibly by inhibiting attachment of virus to cells. On the other hand neither differential induction nor resistance to them appears to be part of the differences in virulence exhibited between Clones 7a and 64d, since the rates of inactivation by their respective nasal inhibitors were very similar (Table I).

There is little doubt from the properties of the acid-treated nasal washes that they contained interferon, as has been reported before (Small *et al.*, 1976). The relation between the rise of interferon and the levels of virus in the nasal washings (Fig.) strongly suggests that interferon contributes to the inhibition of virus-shedding *in vivo*. Our results confirm and extend the work of Small *et al.* (1976) and broadly agree with observations on human and mouse influenza. Thus a temporal relation between the appearance of interferon and recovery from infection is apparent in natural human influenza (Douglas, 1975). Jao *et al.* (1970) showed that interferon appeared in nasal washes of human volunteers after infection with an influenza virus. The duration of virus-shedding after the rise of interferon to peak titres was variable and in some volunteers it persisted beyond the rise and disappearance of interferon. However, interferon appeared to be important in the recovery process since clinical symptoms improved as interferon reached peak titres (Jao *et al.*, 1970). Interferon appeared important in defence against influenza in some studies with mice (Haller *et al.*, 1979; Suzuki *et al.*, 1975; Zee *et al.*, 1979) and the appearance of interferon in tracheobronchial washes was associated with decline of virus titres in the lungs (Iwasaki and Nozima, 1977). However, anti-interferon serum had no effect on infection in another study with mice (Gresser *et al.*, 1976).

Although interferon appears to be a defence mechanism in the upper respiratory tract of ferrets, neither differential induction of it, nor resistance to it, was the reason for the differences in virulence between Clones 7a and 64d. The attenuated Clone 64d induced slightly less interferon and was as resistant to its action, if not more so, than the virulent clone 7a. This agrees with studies in mice where no correlation was found between the virulence of influenza virus strains and interferon production in the lung (McLaren and Potter, 1973; Raut *et al.*, 1975) or their sensitivity to inhibition by interferon (McLaren and Potter, 1973). In humans also, the amount of interferon was directly related to the degree of influenza virus replication or to the severity of illness (Douglas, 1975).

With regard to the origins of the interferon in the nasal washes it may be pertinent that the onset of interferon production coincided with 2 other non-specific host defences, the nasal inflammatory phagocytes and pyrexia (Toms *et al.*, 1977). This suggests that interferon, like the pyrexia (Sweet *et al.*, 1979), may be induced following virus–phagocyte interaction in the upper respiratory tract rather than by the infected epithelial cells. A correlation between interferon levels in the serum of various strains of mice and the ability of leucocytes from these mice to produce interferon *in vitro* has been reported previously (Soloviev, 1968). The temporal relation between fever and interferon production may also be important since interferon activity is potentiated at pyrexial temperatures (Heron and Berg, 1978).

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