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THE LOCAL ORIGIN OF THE FEBRILE RESPONSE INDUCED IN FERRETS DURING RESPIRATORY INFECTION WITH A VIRULENT INFLUENZA VIRUS

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Summary.—Intranasal infection of ferrets with a virulent Clone (7a) of the recombinant influenza virus A/PR/8/34—A/England/939/69 (H_3N_2) produced a fever approximately 24 h in duration beginning about 29 h after infection. The origin of this fever has been investigated as an indication of what might happen in influenza in man.

The systemic production of fever by virus interaction with phagocytes in the reticuloendothelial system appeared unlikely because insufficient virus escaped into the bloodstream. Ten half-hourly i.v. injections of 10^8 50% Egg-Bit Infectious Doses ($EBID_{50}$) of virus were needed to produce a fever of short duration (3–8 h). Yet, after the intranasal infection, which results in the 24 h fever, the total virus content in the nasal mucosa was less than 10^8 $EBID_{50}$ before the onset of fever and only reached $10^{8.5}$ $EBID_{50}$ for 4 h during fever. Also, just before or during the fever produced by intranasal infection, influenza virus antigens could not be detected by fluorescent antibody in the spleens of the animals but were detected in animals receiving a single bloodstream injection of 10^8 $EBID_{50}$ of virus.

Fever is more likely to result from release of leucocyte pyrogen by virus–phagocyte interaction in the upper respiratory tract. A pyrogen active in ferrets with the characteristics of leucocyte (endogenous) pyrogen was produced by incubating influenza virus with ferret peripheral phagocytes *in vitro*. A pyrogen with similar properties was released by incubation of nasal inflammatory cells collected from infected febrile ferrets and many of the cells were shown by fluorescent antibody to have interacted with influenza virus.

INFLUENZA in man is usually a non-lethal infection of the upper respiratory tract with occasional lung involvement and rare viraemia; in addition to respiratory distress the main constitutional effects are pyrexia, headache, anorexia, listlessness and myalgias (Davenport, 1961; Stuart-Harris, 1965). In the ferret a similar respiratory disease results from intranasal inoculation of virus (Stuart-Harris, 1965; Toms *et al.*, 1976) but the only constitutional sympton that can be monitored is pyrexia. However, the animal model affords a means of investigating the relation between pyrexia and virus replication and dissemination, a relation which may hold for human influenza.

Fever, induced by exogenous pyrogens including viruses, is believed to result from their interaction with "professional" phagocytes whereby a leucocyte (endogenous) pyrogen is released. The latter acts on the hypothalamus, *via* synthesis of prostaglandin E, to raise body temperature (Feldberg, 1974; Bodel and Miller, 1977). Although there have been no observations with ferret cells, rabbit polymorphonuclear (PMN) and mononuclear (MN) phagocytes released pyrogen on interaction with influenza virus *in*

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vitro (Kanoh and Kawasaki, 1966; Kawasaki and Kanoh, 1974). Furthermore, a febrile response, with detectable pyrogen in the bloodstream, followed i.v. inoculation of rabbits with large quantities of semi-purified influenza virions (Kosel and Kohlhage, 1970; Wagner, Bennett and Lequire, 1949; Atkins and Huang, 1958). Hence release of endogenous pyrogen is probably the cause of fever production in influenza, but where the main virus–phagocyte interaction occurs is not known. Three possibilities have been suggested by Cranston *et al.* (1971): (a) release of endogenous pyrogen into the bloodstream after interaction of virus with inflammatory cells at the site of virus replication; (b) escape of cells, which have undergone this virus-cell interaction locally, into the bloodstream where the release of pyrogen occurs; and (c) escape of virus into the circulation to interact with PMN or MN phagocytes in tissues such as liver and spleen. Bodel and Hollingsworth (1968) and Murphy (1976) have shown that leucocyte pyrogen can be produced at inflammatory sites and be released into the bloodstream. Also, with regard to influenza, cultures of spleen from rabbits which had been inoculated i.v. with influenza virus released pyrogen *in vitro*, suggesting that if virus escaped from the respiratory tract the necessary phagocyte–virus interactions could occur in the spleen (and probably liver) (Gander, 1973).

Following intranasal infection of ferrets with a virulent clone (7a) of the recombinant virus A/PR/8/34—A/England/939/69 (H_3N_2), fever occurred on the second day and lasted approximately 24 h (Toms *et al.*, 1976, 1977). The onset of fever correlated with the influx of nasal inflammatory cells (both PMN and MN phagocytes) and local phagocyte–virus interactions almost certainly occurred. There was a reduction of virus titres in the nasal washings which correlated with the onset of the inflammatory response (Toms *et al.*, 1977) and a similar reduction of virus was found in virus–ferret-phagocyte interactions *in vitro* (Sweet *et al.*, 1977*a*). With regard to escape of virus from the respiratory tract of ferrets Liu (1955) found influenza virus antigens in the mediastinal lymph node and, after intranasal infection, Toms *et al.* (1976) regularly detected Clone 7a in the cervical lymph node. However, in the latter work, only spasmodic isolations were made from liver, spleen, plasma and kidney. Thus evidence from ferret infection for escape of some virus from the respiratory tract is not as strong as that for man (Naficy, 1963; Stanley and Jackson, 1966; Khakpour, Saidi and Naficy, 1969; Lehmann and Gust, 1971; Wilson *et al.*, 1976; Frankova and Jirasek, 1974; Witzleb *et al.*, 1976). However, an inability to demonstrate systemic virus by infectivity assay may not necessarily mean that large quantities of virus were not released into the bloodstream to elicit fever by interacting with the reticuloendothelial system. Large quantities of infectious virus inoculated intracardially into ferrets disappeared rapidly from the blood and could not be found in the tissues, probably because they were neutralized and sequestrated by the reticuloendothelial system (Toms, Roszstoczy and Smith, 1974).

This paper describes 3 related investigations designed to differentiate between the local and systemic origins of fever. Firstly, the amount of intracardially injected virus that was needed to sustain a fever comparable to that produced by respiratory infection following intranasal inoculation has been related to the total amount of virus available in nasal turbinates during such infection. Secondly, an attempt has been made to estimate the amount of virus antigen released into the bloodstream during respiratory infection by examination of the livers and spleens of such animals, using fluorescent antibody methods, and comparing these with the livers and spleens of animals that had received graded doses of intracardially inoculated virus. Thirdly, attempts have been made to demonstrate that nasal phagocytes from infected ferrets are associated with influenza virus antigen

and release endogenous pyrogen on incubation *in vitro*.

MATERIALS AND METHODS

Influenza virus.—The recombinant virus A/PR/8/34—A/England/939/69 Clone 7a (H_3N_2) and the preparation of virus stocks were described by Sweet, Stephen and Smith (1974*b*) and Gould *et al.* (1972).

Infectivity assays.—The egg-bit assay of Fazekas de St Groth and White (1958) was used as described previously (Sweet, Stephen and Smith, 1974*a*).

Intranasal inoculation of ferrets.—This was performed as described by Toms *et al.* (1976).

Intracardial and intravenous inoculation of ferrets.—Intracardial inoculations were performed as described by Sweet *et al.* (1977*a*). For i.v. inoculation the jugular vein was exposed under pentobarbitone anaesthesia (Sagatal, May and Baker Ltd, i.p. *ca* 0·5 ml/kg body wt) and non-pyrogenic vinyl tubing (ERP 0·5 mm outer bore, 0·25 mm inner bore; ESCO (Rubber) Ltd, London) inserted as described by Popovic and Popovic (1960). The wound was closed with clips (Michel clips, Hauptner Instruments, Brookwick, Ward & Co. Ltd, London), sprayed with plastic dressing (Hibispray, Avelex Ltd, Wigan, Lancs.) and covered with adhesive plaster strapping leaving access to the tubing. A 5ml blood sample was removed *via* the cannula, which was then filled with PBS (A) (Dulbecco A, Oxoid) and sealed with a closed 23G needle (Luer lock fitting removed) and taped down. The animals were left for 1–2 days to recover, then the closed 23G needle was removed, a 23G–1¼ in needle inserted and the sample inoculated into the ferret via the tubing. The cannula was washed by inoculation of a further 0·5 ml of PBS(A) and resealed. Inoculations could be performed at regular intervals without anaesthesia.

Rectal temperatures.—These were taken and the febrile response defined as described by Toms *et al.* (1976).

Measurement of virus infectivity in macerates of nasal turbinates.—This was performed as described by Sweet, Stephen and Smith (1974*c*).

Detection of influenza antigen in spleen and nasal exudate cells by the fluorescent antibody technique.—Segments of spleen were frozen in a Slee carbon dioxide freezing unit and 5–6μm sections obtained on a cryostat. Nasal exudate cells, from infected and non-infected ferrets, collected as described below, were resuspended to similar concentration (10^6 cells/ml) and drops were spread, dried and fixed in acetone as described by Kingsman, Toms and Smith (1977). The presence of viral antigen was determined by indirect immunofluorescence using hyperimmune rabbit antiserum as described by Kingsman *et al.* (1977), except that it was raised to Clone 7a influenza virus. This antiserum contained antibody to both internal and surface antigens of influenza virus.

Exclusion of exogenous pyrogen.—All solutions used in pyrogen experiments were autoclaved for 2 h. Glassware was sterilized at 170° for at least 2 h. Only non-pyrogenic polypropylene syringes and needles (Becton, Dickinson & Co. Ltd, Ireland) were used.

Preparation of leucocyte pyrogen from ferret peripheral white blood cells.—The blood cells, removed by centrifugation (750 *g*, 15 min, 4°) from approximately 100 ml of citrated ferret blood, were treated with 2–3 times their packed cell volume of 3% (w/v) dextran (No. D-5001, Sigma Chemical Co.) in citrated saline. After standing for 30–40 min at 4° the supernatant was removed from the settled red blood cells and centrifuged (750 *g*, 15 min, 4°) to remove the white cells, which were washed once with PBS(A) before being resuspended in 1 ml of 0·85% saline. Contaminating red blood cells were lysed by the addition of 3 ml of sterile distilled water for 30 sec, and then the isotonicity was restored by the addition of 1 ml of 3·5% saline. The white cells were centrifuged and washed 3 times in Eagle's MEM (Wellcome Reagents Ltd). Finally they were resuspended in Eagle's MEM+10% (v/v) heat-inactivated (56°, 1 h) normal ferret serum to give a final concentration of 10^8 cells in 1 ml. To this suspension was added either 10^{10} $EBID_{50}$ of Clone 7a in 0·5ml Eagle's MEM or the medium alone. The mixtures were incubated for 1 h at 37° before adding further Eagle's MEM+10% ferret serum to give a final concentration of 10^7 cells/ml. After a further incubation for 15–17 h at 37° the cells were removed by centrifugation (750 *g*, 15 min, 4°) and the supernatant stored at −70°. The material was inoculated into ferrets *via* an i.v. cannula at a dose of 2 ml/kg.

Preparation of leucocyte pyrogen from nasal exudate cells.—Five ferrets were infected with 10^6 $EBID_{50}$ Clone 7a and, at hourly intervals from 36 to 40 h after infection, when both nasal cell counts and rectal temperatures were rising, nasal washings were made with PBS(A) as described by Toms *et al.* (1977). The inflammatory cells in these washings were removed by centrifugation (350 *g*, 10 min, 4°) and resuspended in 0·85% saline to give approximately 10 ml containing $5–7 \times 10^6$ cells/ml. The suspension was incubated at 37° for 2 h, when the cells were removed by centrifugation and the supernatant stored at −70°. The material was inoculated into ferrets *via* an i.v. cannula at a dose of 2 ml/kg.

Bacterial endotoxin.—A commercial preparation (Sigma Chemical Co.) of lipopolysaccharide (Product No. L-3129) from *E. coli* (serotype No.

0127: B8) was used dissolved in 0·85% sterile saline.

RESULTS

Total virus infectivity in nasal turbinates of ferrets at time of pyrexia following intranasal inoculation

The total infectious virus contents of the nasal turbinates of groups of 4 ferrets killed at 4-hourly intervals after intranasal inoculation of 10^6 $EBID_{50}$ of virus are shown in Fig. 1 in relation to the pyrexia produced during the respiratory infection. From 20 to 40 h after infection, which is the period of peak virus titres in nasal washes (Toms *et al.*, 1977), total virus titres always exceeded $10^{7·5}$ $EBID_{50}$ but were never greater than $10^{8·5}$ $EBID_{50}$, and then only over a 4h period. The febrile response did not occur until nasal titres began to rise above $10^{7·5}$ $EBID_{50}$.

Extent and duration of fever produced by bloodstream inoculation of virus

After intranasal inoculation of ferrets with Clone 7a, fever occurred at approximately 30 h, reached a peak of 2–3° above the pre-infection mean and lasted for approximately 24 h (Fig. 1; Toms *et al.*, 1977). The pyrexia produced by single and multiple intracardial or i.v. inoculations of virus is described below.

Single intracardial inoculation of virus

Ferrets received 10^8 or 10^9 $EBID_{50}$ of live virus in 0·5 ml of PBS(A). At least 10^8 $EBID_{50}$ was required to raise the rectal temperature more than 1° above the pre-injection mean (Table I). Only with 2 of 6 animals inoculated with 10^8 $EBID_{50}$ was the fever observed and this was of short duration ($\leqslant 1$ h), occurring 7–9 h after inoculation. With 10^9 $EBID_{50}$ fever occurred approximately 5 h after inoculation and lasted for 3–5 h (Table I). Control inoculations of PBS(A), egg-allantoic fluid and inoculation with 10^7 $EBID_{50}$ virus failed to elicit a pyrexia.

Multiple i.v. inoculations of virus

The rectal temperatures of ferrets following 10 i.v. inoculations at half-hourly intervals of 10^9 $EBID_{50}$, 10^8 $EBID_{50}$, 10^7 $EBID_{50}$ live virus or 10^9 $EBID_{50}$ heat-inactivated virus (56°, 2 h) are summarized in Table I. With 10^9 $EBID_{50}$ live virus the pyrexia occurred between 2 and 6 h after the first injection, attained a maximum (about 3·9° above the pre-injection mean) above that produced by a single inoculation, and was more prolonged (Table I). Ten injections of 10^8 $EBID_{50}$ live virus produced a fever similar in duration and height to that of a single injection of 10^9 $EBID_{50}$ (Table I).

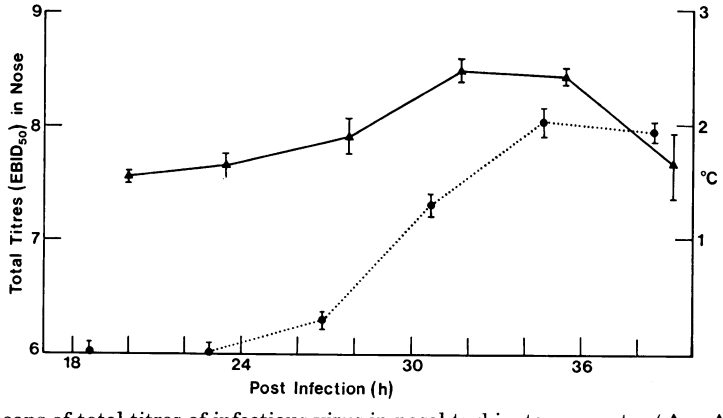

FIG. 1.—The means of total titres of infectious virus in nasal turbinate macerates (▲—▲) and mean rises in rectal temperatures above the pre-infection mean for each animal (●···●), of animals inoculated intranasally with 10^6 $EBID_{50}$ Clone 7a. Each point represents the mean (bar the s.e. mean) of readings from 4 animals.

TABLE I.—*Minimum Fever-inducing Dose Following Single or Multiple Intracardial Inoculation of Influenza Virus Clone 7a*

Inoculum ($EBID_{50}$)	No. of responders*	Time of onset: h after first inoculation	Fever duration (h)	Maximum rise in temperature (°C)
Single dose				
10^8	2/6	7–9	⩽1	1·3
10^9	5/5	5	3–5	2·9
Multiple doses†				
10^7	3/3	11–14	⩽1	1·4
10^8	4/5	5–11	3–8	3·1
10^9	5/5	2–6	⩾13<24	3·9
10^9 killed	0/2	–	–	–

* No. of animals showing temperature rise ⩾1° over preinjection mean / No. of animals tested

† 10 i.v. inoculations at half-hourly intervals.

–, no fever response.

Ten injections of 10^7 $EBID_{50}$ live virus hardly produced a significant response and certainly no more than a single injection of 10^8 $EBID_{50}$ live virus. Multiple injections of heat-killed virus (10^9 $EBID_{50}$) elicited no significant fever response.

Fluorescent antibody staining of spleens of ferrets infected intranasally and after intracardial inoculation of graded doses of influenza virus

Sections of spleens from the 4 animals killed at each time point after intranasal inoculation in the experiment summarized in Fig. 1 were examined for influenza virus antigens by fluorescent antibody techniques; no cells showed positive immunofluorescence at any time point from 20 to 40 h (nor could infective virus be isolated from blood clot, serum or homogenates of spleen and liver). In contrast, antigen could be detected in the spleens of animals killed ½ h after a single i.v. inoculation with 10^9 or 10^8 $EBID_{50}$ of virus (Table II); antigen could not be detected after inoculation of 10^7 $EBID_{50}$. Sections of livers were also examined but antigen could not be detected as readily as with spleen.

TABLE II.—*Influenza Virus Antigen Detected by Fluorescent Antibody Following Intracardial Inoculation of Graded Doses of Clone 7a*

Dose ($EBID_{50}$)	No. of responders*	Magnitude of response in animal number		
		1	2	3
10^9	3/3	+++	+++	+++
10^8	2/3	+	+	—
10^7	0/3	—	—	—

* No. of animals showing fluorescent cells in spleen sections / No. of animals tested

—, no immunofluoresence positive (IF+) cells seen.

+, <5 IF+ cells seen in a section.

+++, many cells clearly IF+.

Pyrogen production following virus–blood-phagocyte interaction in vitro

Virus–cell interaction *in vitro* (see Materials and Methods) released a pyrogen with the characteristics of "leucocyte pyrogen". Animals inoculated with this pyrogen rapidly produced a fever; they were febrile 15 min after injection, the fever peaking at 30 min and disappearing in 2–3 h (Fig. 2a). Pyrogen was not produced by cells incubated without virus (Fig. 2b) and heating the virus-induced pyrogen at 56° for 2 h before the animal was inoculated completely removed its activity (Fig. 2c). The response of the ferrets to the "leucocyte pyrogen" was too rapid for it to have been due to any endotoxin which might have been present in the preparation; the response of ferrets to the latter did not occur until 45–60 min

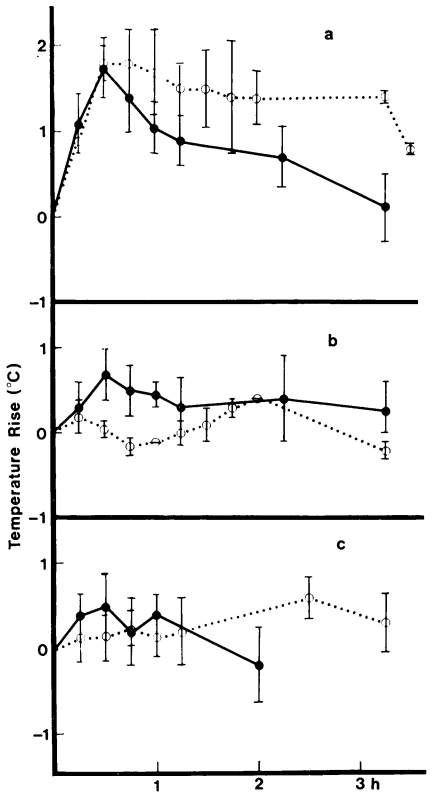

FIG. 2.—Mean rises in rectal temperatures above the pre-injection mean for animals inoculated i.v. with 2 ml/kg: (a) leucocyte pyrogen derived from the *in-vitro* interaction of ferret peripheral blood leucocytes with influenza virus (see Materials and Methods); (b) control supernatant from cells incubated without virus; or (c) heated (56°, 2 h) leucocyte pyrogen. Each curve (○···○, ●—●) shows the result of a different batch of material and is the mean (bar the s.e. mean) of readings in 2–3 animals.

after injection and lasted for 3½ (0·02 μg/kg; Fig. 3a) to 7 h (2 μg/kg; Fig. 3b).

Pyrogen production by nasal inflammatory cells harvested from infected ferrets and incubated in vitro

Nasal exudate cells consisted predominantly of PMN phagocytes with some MN phagocytes and ciliated epithelial cells as described previously (Toms *et al.*, 1977). Fluorescent antibody observations with appropriate controls showed in 2 experiments that influenza virus antigens

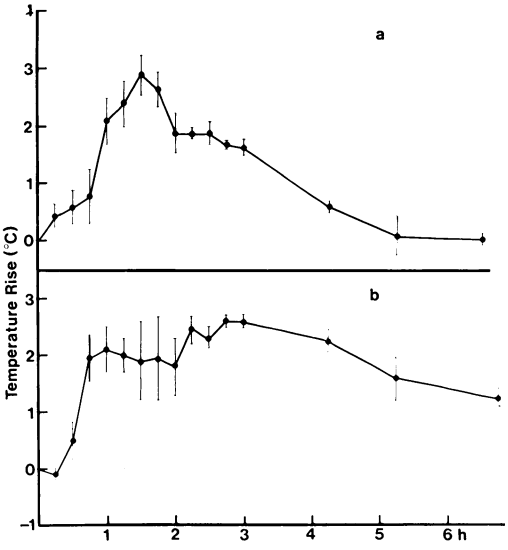

FIG. 3.—Mean rises in rectal temperature above the pre-injection mean for animals inoculated i.v. with: (a) 0·02 μg/kg; or (b) 2 μg/kg endotoxin. Each point is the mean (bar the s.e. mean) of readings in 2–4 animals.

were associated with the majority of these cells (Fig. 4), thus confirming that virus–phagocyte interaction had occurred *in vivo*. These infected cells released a pyrogen with the characteristics of leucocyte pyrogen on incubation *in vitro* (Fig. 5a). As with the product produced by virus–cell interaction *in vitro*, this pyrogen was inactivated by heating at 56° for 2 h (Fig. 5b).

DISCUSSION

The 24h fever occurring after intranasal infection of ferrets with Clone 7a might have been caused by virus interaction with phagocytes in the reticuloendothelial system. However, virus escape from the infected upper respiratory tract appeared insufficient for this purpose. A bloodstream inoculation of 10^8 $EBID_{50}$ of virus was required to induce any fever and 10 inoculations of 10^8 $EBID_{50}$ over 5 h were needed to produce a 3–8h fever (Table I). Thus it appears that a viraemia corresponding to this quantity of virus would be needed for approximately 24 h to induce the 24h fever observed in ferrets infected

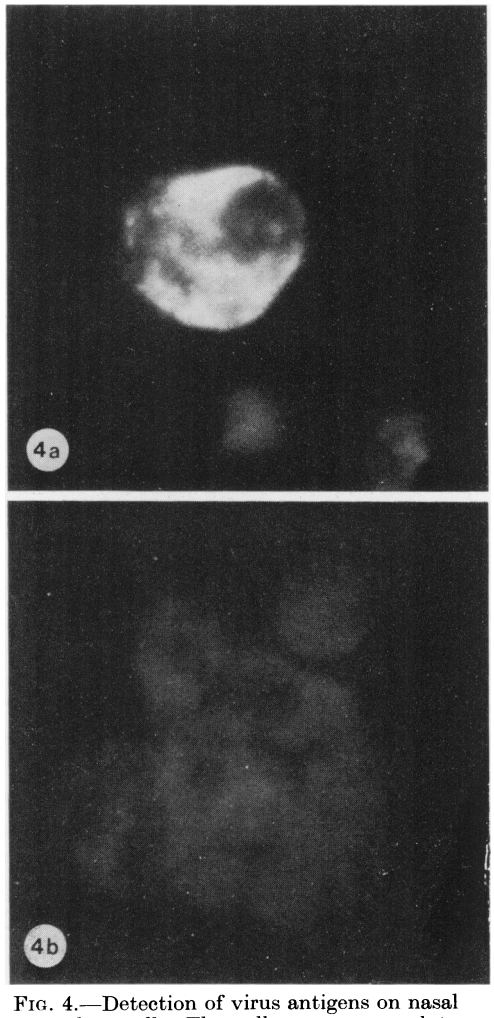

FIG. 4.—Detection of virus antigens on nasal exudate cells. The cells were exposed to rabbit anti-7a or normal rabbit serum followed by FITC conjugated sheep anti-rabbit immunoglobulin plus Rhodamine B-200. (a, b) cells from infected ferrets exposed to: (a) rabbit anti-7a serum; (b) normal rabbit serum. Cells from non-infected animals exposed to rabbit anti-7a serum (not shown) were similar to those in Fig. 4b.

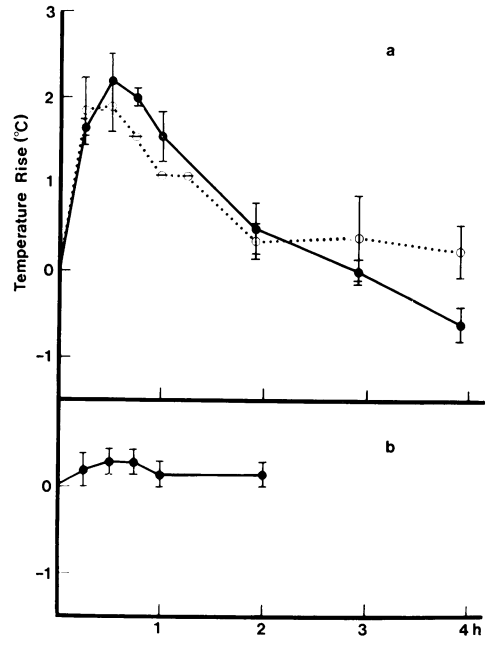

FIG. 5.—Mean rises in rectal temperatures above the pre-injection mean for animals inoculated i.v. with leucocyte pyrogen (2 ml/kg) derived from the *in-vitro* incubation of nasal inflammatory exudate cells obtained from ferrets infected with influenza virus (see Materials and Methods); (a) unheated preparation, (b) heated (56°, 2 h) preparation. Each curve (○···○, ●—●) shows the result of a different batch of material and is the mean (bar the s.e. mean) of readings in 2–3 animals.

intranasally with Clone 7a. However, total virus titres in the nasal mucosa were below 10^8 $EBID_{50}$ before the onset of fever and only exceeded this figure ($10^{8\cdot5}$ $EBID_{50}$) for a 4h period during the fever (Fig. 1). All of this virus would have to be released continuously into the bloodstream over a 24h period to produce the fever by systemic reactions. This is improbable, and 2 lines of evidence tend to substantiate this view. Firstly, the inability to detect influenza virus antigen by fluorescent antibody in the spleens of infected animals examined in the period immediately before and during the fever, while such antigens could be detected in ferrets given a single i.v. inoculation of 10^8 $EBID_{50}$ virus, suggests that escape of this level of infectious or non-infectious virus does not occur. Secondly, previous work with pregnant ferrets (Sweet, Toms and Smith, 1977*b*) showed that an intracardial inoculation of such animals with $10^{7\cdot4}$ $EBID_{50}$ virus led to foetal membrane infection, but infection of these tissues did not occur following intranasal infection, which suggests that

virus release from the upper respiratory tract was less than $10^{7.4}$ $EBID_{50}$.

With regard to the local production of endogenous pyrogen by nasal inflammatory cells, virus appeared to have interacted with these cells *in vivo* because viral antigen was found associated with them by fluorescent antibody techniques. Furthermore, after experiments *in vitro* had shown that a pyrogen with the characteristics of leucocyte pyrogen was liberated from ferret blood phagocytes after interaction with influenza virus, the release of a similar pyrogen was detected when nasal leucocytes collected from infected ferrets were incubated *in vitro*. Thus, leucocyte pyrogen is almost certainly formed in the infected nasal tract and there are the following strong indications that its production and action occurs over a sufficiently long period to account for the 24h pyrexia. Firstly, during and immediately before the fever, virus and inflammatory cells were present in the nasal tract for a continuous interaction to take place (Toms *et al.*, 1977). Secondly, pyrogen release from phagocytes *in vitro* begins about 2 h after stimulation of the leucocytes by phagocytosis and continues for at least 18 h; Atkins and Bodel (1971) described this continuous release for human phagocytes and in the present work pyrogen was found after 16–18h interaction of influenza virus with ferret phagocytes *in vitro*. Finally, in ferrets infected with Clone 7a, a statistical correlation existed between cell counts in the nasal wash at 21 h after infection and rise in rectal temperatures at 29 h after infection; and this correlation between cell counts and subsequent rise in rectal temperature persisted for 24 h (Toms *et al.*, 1977).

Thus the evidence suggests that the fever induced in ferrets during influenza results from the local interaction of virus with phagocytes in the upper respiratory tract, with the resultant release of pyrogen into the bloodstream. However, 2 further alternatives should be mentioned which relate to the local rather than systemic origin of fever. The leucocytes "primed" by interaction with virus in the upper respiratory tract may themselves enter the bloodstream, there to release their pyrogen, as suggested by Cranston *et al.* (1971). Alternatively, virus has been isolated from ferret cervical lymph nodes 2 days after infection when fever first appears (Toms *et al.*, 1976) and it is possible that virus–phagocyte interactions occur here as well as in the respiratory tract.

Finally, it is intriguing to speculate how an infection limited to the respiratory tract could produce a wide range of constitutional symptoms. This has been loosely attributed to "toxic" products of tissue destruction. However, all the symptoms of influenza such as shivering, malaise, headache, and frequently nausea and polymyalgia have followed the i.v. administration of autologous endogenous pyrogen in man (Rawlins and Cranston, 1973). Most of the constitutional effects of influenza may, therefore, be explained solely by the local interaction of virus with phagocytes of the respiratory tract releasing endogenous pyrogen into the bloodstream.

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