# Serum Interferon in Bovine Ephemeral Fever

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by

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The object of the development of an assay system for interferons in the blood of ephemeral fever cases was to relate its appearance and disappearance to clinical signs of ephemeral fever. Ms Zakrzewski adapted a methodology and carried out the assays. Dr Uren and I made the clinical observations and collected most of the blood sampling. I wrote the paper. The head of the laboratory would not pass the paper for publication as we failed to isolate BEF virus from the sick cattle. At the time, the isolation methods gave very erratic results. There was no opportunity to repeat the assay some years later when better methods were developed for virus isolation and interferon assay.

T.D. St.George Date 12-2-02

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# Serum Interferon in Bovine Ephemeral Fever

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Running Title: Interferon in bovine ephemeral fever.

***Abstract***

Ephemeral fever was produced experimentally in eight cows. Clinical signs were apparent 80 to 130 h after inoculation and blood samples were subsequently collected at hourly intervals. Recovery followed 34 to 82 h after the onset of fever. Neutralising antibodies were first detected in serum 72 to 96 h after recovery. Plasma fibrinogen levels rose from a mean of 59 ± 21 $mg/l^{-1}$ to a peak of 153 ± 44 $mg/l^{-1}$, 56 to 96 h after the initial rectal temperature rise. Interferon was detected in serum approximately 8 - 14 h before the temperature and neutrophils began to rise and reached a peak 10 h later. The level of interferon fell before clinical recovery. Increased levels of interferon were detected in seven natural cases of ephemeral fever.

ADDITIONAL KEY WORDS: neutrophilia, lymphopaenia, fibrinogen.

## INTRODUCTION

Ephemeral fever of cattle is caused by the insect-borne rhabdovirus, bovine ephemeral fever (BEF) virus (van der Westhuizen *et al.* 1967). Mackerras *et al.* (1940), van der Westhuizen *et al.* (1967) and many others demonstrated that BEF virus circulated in the blood stream of cattle during illness, though the times of onset and duration of viraemia were uncertain. Mackerras *et al.* (1940) showed that the infectivity of BEF virus lay in the leucocytic fraction of bovine blood. Subsequently, Theodoridis (1969) confirmed this and Young and Spradbrow (1977, 1980) observed virus in neutrophils in the blood stream and tissues. The pathology of the disease with systemic inflammation was described by Mackerras *et al.* (1940) and Basson et al. (1970). The biochemical indicator, serum fibrinogen rises to high levels during disease confirming that inflammation has occurred (St. George *et al.* 1984). However, the pathogenesis of the disease is poorly understood and our study was undertaken to determine when interferon was present in the bloodstream in relation to the clinical signs, the viraemia, and the haematological and biochemical changes of ephemeral fever in cattle.

## MATERIALS AND METHODS

The production of interferon in cattle during ephemeral fever was studied in experimentally and naturally infected cattle. The experimentally infected cattle were held in insect proof isolation units and the naturally infected cattle were studied on a farm during an epidemic of ephemeral fever. Similar methods for examination, sampling and laboratory procedures were used for all cattle.

*Viruses*

The stock of BEF virus used for inoculation of experimental cattle was a heparinized whole blood suspension obtained from a steer previously infected with a field isolate of BEF virus. The virus used was the 11th cattle passage after natural disease. The virus used in the neutralization tests was strain BB7721, originating from the same steer, isolated in mice by Doherty *et al.* (1969), then adapted to BHK21 tissue cultures.

Newcastle disease virus (NDV) strain V4 (Simmons 1967) was used for interferon assay at the 9th egg passage. The stock of virus, which was diluted with an equal volume of glycerol, exhibited a haemagglutin (HA) titre of 320 and was stored at -20°C.

*Haematology*

Total leucocyte counts were obtained from whole blood mixed with Ethylene Diamine Tetracetic Acid Disodium Salt (EDTA) using a Coulter Counter model Dn (Coulter Electronics Ltd, Harpenden, England). A blood smear was made from the uncoagulated blood within half an hour of collection, air dried, and fixed with methyl alcohol. Differential leucocyte numbers were determined by staining the blood smears with May-Grunwald and Giemsa and examining cells under a light microscope. Absolute numbers of each type of cell were calculated from the total leucocyte count.

*Virus Isolation*

A proportion of the blood collected into EDTA was centrifuged at 1500 g for 10 min to separate the leucocyte fraction. This was then cultured for bovine ephemeral fever virus as described by St.George (1985). In brief, approximately 0.1 ml of the leucocyte fraction was inoculated into Aedes albopictus (Singh) tissue cultures and then subcultured into baby hamster kidney (BHK21) monolayers after one week's incubation at 26°C. The BHK21 monolayers were observed for cytopathology three times per week, and subcultured once.

*Biochemistry*

Plasma was obtained by centrifugation of uncoagulated whole blood at 1000 g for 10 min and then diluted with an approximately equal volume of 0.025 M $CaCl_2$ solution. Plasma fibrinogen levels were determined by the clot weight method (Ingram 1952).

*Tissue culture for interferon assay*

Primary calf kidney (BK) cells were used initially and later bovine kidney cell line (MDBK) (Flow Laboratories, Sydney, Australia) (Madin and Darby 1958) were found equally satisfactory for the assay of bovine interferon. These cells were grown in 175 $cm^2$ tissue culture flasks (A/S Nunc, Kamstrup, Denmark)

seeded at 10 x $10^6$ cells/flask, incubated at 37°C and subcultured at weekly intervals. The growth medium for BK and MDBK was (1) Hanks' balanced salt solution plus 0.5% lactalbumin, 0.01% yeast extract, pH 7.4 and 10% foetal calf serum (FCS) (Gibco Australia Pty Ltd, Melbourne, Australia) and (2) Eagles minimum essential medium with Earle's salts (Gibco Australia Pty Ltd, Melbourne, Australia) plus 2 mM glutamine, 1% nonessential amino acids, 0.85 g/l sodium bicarbonate, pH 7.2 (MEM) and 10% FCS, respectively.

Interferon assay was adapted from the method using Sendai virus described by Hahon (1981) and was carried out in flat bottom 96 well tissue culture plates (A/S Nunc, Kamstrup, Denmark). The growth medium for BK and MDBK was (1) Medium 199 with Hanks' salts (Gibco Australia Pty Ltd, Melbourne, Australia) 2 mM glutamine 1.12 g/l sodium bicarbonate, pH 7.0 and 10% FCS and (2) MEM and 10% FCS, respectively. Individual serum samples were diluted at 1/4 in the first well and serially titrated two-fold in the plate (100 ml/well). An equal volume of cells at a concentration of 3 x $10^5$ cells/ml was added to each well. Serum, cell and virus controls were included in the assay. The plates were covered with plate sealers and incubated at 37°C and 5% concentration of carbon dioxide in air until confluent cell monolayers had grown on the bottom of the wells for 24 h. The media was decanted by inverting the plates which were then blotted dry. The cell sheets were washed once with a sterile phosphate buffered saline (PBS) solution (pH 7.2), the washings decanted and a volume of 50 µl/well of NDV was added at a concentration of 1 HA unit/ml. The virus was adsorbed at 37°C/5% $CO_2$ for 1 h after which time the virus was decanted, plate blotted, a volume of 200 ml of growth medium added to each well and re-incubated at 37°C/5% $CO_2$ for a further 40 h. Guinea pig erythrocytes which had been washed twice in cold PBS were used to indicate haemadsorption. A 0.4% guinea pig erythrocyte suspension was prepared and a volume of 50 ml/well was added. The plates were incubated at 25°C for 30 min, washed thoroughly with cold PBS to remove excess erythrocytes, and examined on an inverted light microscope. The end point of interferon activity was taken as that showing a 50% reduction of the number of haemadsorbing cells, compared with that exhibited in the virus control. For each dilution series four wells were used per dilution of serum to be assayed. All serum samples from a particular cow were examined in a single test. A bovine serum which exhibited a capacity to interfere with the replication of NDV in the assay and one which showed no activity in preliminary tests were included in each subsequent test.

*Characterization of interferon*

Serum samples were collected from animals before inoculation with ephemeral fever and at the peak of the interferon titre. Aliquots were treated as follows: the pH was adjusted to pH 2 and pH 4 by the addition of 1N HCl at 10 min intervals for 2 h, and at 19 h the pH was returned to pH 7.2 by the addition of an appropriate volume of 1N NaOH. Other aliquots of the same sera were held

in sealed glass ampoules in a water bath at 56°C and were removed at 10 min intervals and immediately stored at -20°C until assayed for interferon.

A serum with a high interferon activity (titre of 2048) was selected and applied to a 96 cm x 1.6 cm Sephacryl G200 column buffered with PBS pH 7.4 plus 0.5M NaC1. Two ml of serum was applied to the column with buffer at a flow rate of 10 ml per hour and 1.7 ml fractions were collected. The optical density of each fraction was measured at 265nm. Each fraction was assayed for interferon activity.

*Experimental Cases - Experiment 1*

Four cattle were obtained from Victoria, an area free of infection with BEF virus. Blood samples were collected three times in the 3 weeks before the experiment commenced and immediately before inoculation.

After inoculation, each animal was observed once daily for 72 h, then hourly for 72 h, then each 6 h for 3 days, then again once daily for 5 days. At each examination, the rectal temperature, respiratory rate and heart rate were recorded and any clinical signs noted. Blood samples were collected for biochemistry, haematology, and serum. Serum was stored at -20°C until assayed for interferon or antibodies.

*Experimental Cases - Experiment 2*

Four similar cattle were inoculated intravenously with blood of the same batch as in experiment 1. They were observed and sampled as in experiment 1. Two other steers were observed and sampled but were not infected with BEF virus.

*Natural cases*

Natural cases occurred during an epidemic of ephemeral fever between 29 January and 15 February 1976 on a dairy farm at Peachester located in south eastern Queensland. A description of the epidemic and the sampling methods was given by St.George *et al.* (1984). Blood samples were collected daily for at least 30 days before and for 14 days after natural ephemeral fever occurred in a very mild to moderately severe form in 10 of the 12 sentinel cattle. Aliquots of serum held at -20°C since collection in 1976 were used for interferon estimations. The interferon levels in seven of the twelve cattle for one week before, during and after clinical illness were estimated.

## RESULTS

*Clinical Signs - Experiment 1*

The four cattle inoculated with BEF developed clinical signs of ephemeral fever. The first signs were observed 100 to 130 h after infection. A rise in rectal temperature began at or shortly before the same time as the first almost imperceptible changes were noted in the behaviour of the cattle. An increase in the pulse rate followed. The clinical signs in the first fever cycle were depression, inclination to rest and some muscular fibrillation in the flanks. The more characteristic signs of stiffness, lameness, anorexia and constipation were pronounced in the second phase of fever. Three of the cattle which became sick remained on their feet, or rose to their feet while being sampled whereas one cow remained quietly in sternal recumbency once fever was established throughout the intensive observation period. The temperature response, haematology and interferon levels for this animal are shown in Figure 1.

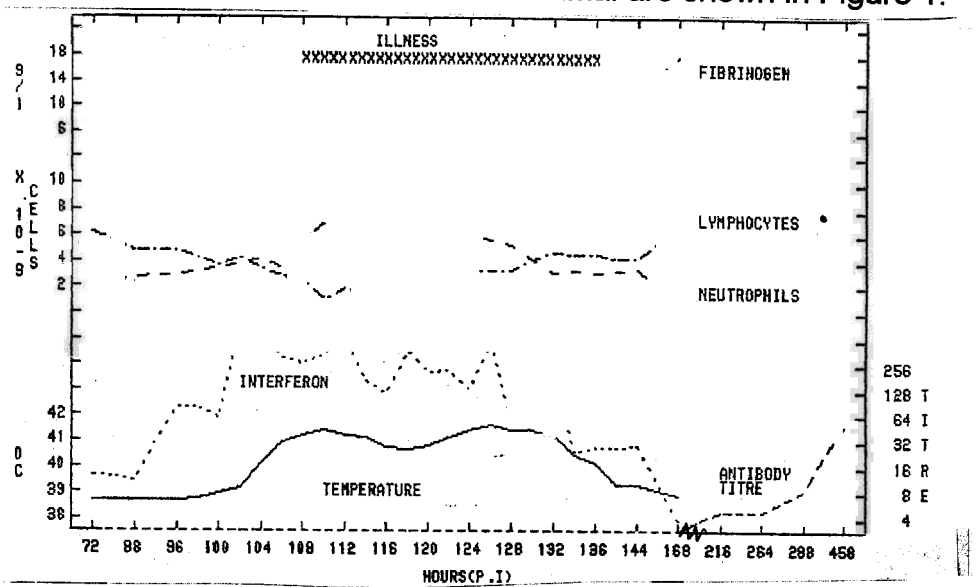


Figure 1. The interferon response is shown in relation to haematology, fibrinogen levels and neutralizing antibody development in the serum of a cow experimentally infected with ephemeral fever virus. Interferon became detectable before fever, illness, or inflammatory response reflected by a rise in neutrophil numbers and in plsma fibrinogen. The irregular interferon levels may be due to assay error as they occurred in series from the other cows. However, 24 of 26 peaks are at intervals of 6 or 8 h.

*Clinical Signs - Experiment 2*

The four cattle in this group developed clinical signs of ephemeral fever 96 to 130 h after inoculation. In the early stages of hourly examination and sampling these cattle were active, but became depressed and recumbent 24 to 36 h after the first onset of fever. The two steers not infected with BEF virus remained normal throughout.

*Neutralizing antibody*

The eight cattle inoculated experimentally with BEF virus developed detectable antibodies to BEF virus 72 to 96 h after their rectal temperature had returned to normal. The uninfected steers in experiment 2 did not develop neutralizing antibodies.

*Haematology*

The eight cattle which responded to experimental infection showed a relative neutrophilia at the time of peak temperature response. A 5-fold increase was observed in one steer and the remaining animals showed from 2- to 4-fold increases in neutrophil numbers. In each case the neutrophilia was characterized by the appearance of up to 30% band forms. All infected cattle showed a fall in lymphocyte numbers, which began prior to any clinical signs or temperature rise being noted. Leucocyte numbers returned to normal values within 48 h. The clotted blood collected after fever began changed in character. Contraction of the clot even 24-72 h after collection was poor and there was a network of fibrin in both the serum and the poorly separated erythrocyte fraction.

This fibrin gave an overall whitish appearance to the outside of the clot. The serum yield was much reduced and some samples gelled. This effect persisted for three days and was similar to that seen in natural cases.

*Virus Isolation*

No cytopathogenic virus was isolated in tissue cultures from any blood sample from the experimental cattle, but was isolated from three of the seven natural cases.

*Biochemistry*

Plasma fibrinogen levels began to rise during the day of peak temperature, the earliest response being recorded 2 h following the initial temperature rise. One steer showed a 3-fold increase in normal values 82 h after the initial temperature rise. The plasma fibrinogen rose from a normal mean of 5.9 ± 2.1 $mg/l^{-1}$ to a peak of 15.3 ± 4.4 $mg/l^{-1}$. The peak response was between 56 h and 96 h after the initial temperature rise. An example of the rise in plasma fibrinogen values is shown in Fig. 3. The fibrinogen values remained elevated for 6 days following the febrile response at which time plasma sampling was discontinued.

*Interferon levels*

A unit of interferon is defined as the reciprocal of the dilution of an interferon preparation which can reduce replication of a sensitive virus by a given percent (Stewart II 1981).

No interferon was detected in the serum of the cattle which did not experience ephemeral fever or in 8 to 25 samples of individual cows prior to the first positive samples.

All cattle which showed clinical signs of ephemeral fever after experimental infection developed high titres of interferon (Table 1). Interferon was first detected in the 1 : 8 dilution of serum in samples collected 8-14 h before a temperature rise began. The series of samples from one of the cows was incomplete at the critical time when the fever began so analysis was carried out on seven of the series. The point at which the rectal temperature began to rise was arbitrarily taken as zero so that the results from the seven cows were initially considered for statistical analysis.

**Table 1. Interferon levels of serum in cows infected with bovine ephemeral fever virus**

| Cow number | Interferon before fever | Maximum titre | Interferon duration hr |
|---|---|---|---|
| 1 | 8 | 350 | 52 |
| 2 | 14 | 1620 | 88 |
| 3 | 8 | 510 | 126 |
| 4 | 12 | 620 | 66 |
| 5 | 2 | 1410 | 40 |
| 6 | 14 | 610 | 72 |
| 7 | 10 | 2830 | 96 |
| 8 | 8 | 4100 | 40 |

However, a second cow had a different febrile pattern from the others in that there was a period of 4 h when the temperature was normal between two episodes of fever. This animal also had the lowest interferon response. The pattern of arrival of the first episode of fever was similar to each of the other six. A statistical analysis therefore was made of the relationship between the rise in temperature and the rise in titre of serum interferon. A composite interferon variable was constructed with an initial 4 h time lag in which the lag was reduced 1 h after every 2 h until it reached zero. There was no lag after hour 13. This model gave a reasonable fit for the six animals. The correlations were 0.38, 0.56, 0.69, 0.75, 0.78 and 0.79. The conclusion by the statistician was that the model does not provide a definitive test that interferon influences temperature, but does indicate that the data does support such a model.

*Interferon Assay and Characterisation*

The bovine sera under test had an effect on the density of growth of the bovine tissue culture cells when used at lower final dilutions. Consistent results

could not be obtained in the assay system unless the serum to be assayed was diluted to 1:8 or greater. This dilution was adopted as the minimum assay level once preliminary experiments had established this point. The effect of pH 2, pH 4 and 56°C with time of treatment is illustrated in Figure 2.

**FIGURE 2**

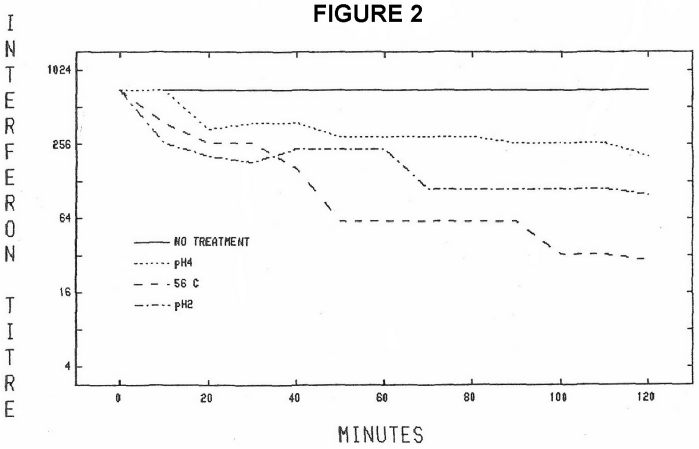


Figure 2. The effect of pH 2, pH 4 and 56°C on interferon in bovine serum at that pH is illustrated in relation to time of exposure.

The fractionated serum showed that the interferon activity occurred in fractions of smaller molecular size than that of the globulin and albumen fractions (Fig.3).

**FIGURE 3**

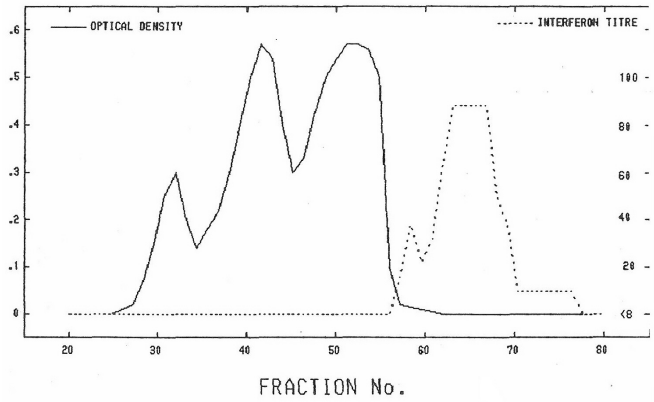


Figure 3. A serum with high interferon activity was applied to a Sephacryl G200 column. The optical density of each fraction was measured a 265nm and each fraction titrated for interferon activity. The molecular weight of all the fractions showing interferon activity was less than that of albumen and globulins.

## *Natural cases*

The seven cattle whose serum interferon was assayed had characteristic clinical ephemeral fever of 2-3 days duration. Interferon was detected in the

serum of each animal on the first day fever commenced, that is, the day before the other characteristic clinical signs of ephemeral fever were noted. The titre rose to a peak of 1024 to 3200 on the first day of clinical disease and declined on the second day to 64 to 256. Thereafter, interferon was not detectable in serum diluted 1:8 from any of the recovering animals.

## DISCUSSION

The experimental cases exhibited clinical signs characteristic of moderately severe ephemeral fever. No cow became temporarily or permanently paralysed. The haemotology and rise in plasma fibrinogen was characteristic of ephemeral fever (St.George *et al.* 1984). Neutralizing antibodies to BEF virus were detected on 7-9 d after inoculation with BEF virus.

The failure to detect BEF virus in the experimental cases by a method which had been successful in isolating five other strains from eight field cases in 1982 (St.George 1985) and three field cases from which blood had been stored since 1976, meant that no comparison could be made between the time of arrival of BEF virus in the blood stream and the rise in serum levels of interferon. The fault lay in the method of virus isolation which would detect only cytopathogenic BEF virus. This became apparent later in three other series of experiments on cattle using the same inoculum (Uren, *et al.* (1989). In these later series, an indirect immunofluorescence test demonstrated the strain of BEF virus used in these experiments was non-cytopathogenic.

The interferon assay had no absolute control, because no standardized bovine interferon was available in Australia, or from the U.S.A. or England at the time the assays were carried out. The method used was adapted from one described by Hahon (1981) which used Chang human conjunctival cells infected with Sendai virus. The bovine cells were used for this assay in order to use cells of the homologous species. Homologous cells were considered to be more satisfactory for interferon production than heterologous cells by Stewart II (1981) though Letchworth and Carmichael (1983) do not consider that this quality necessarily applies to bovine interferon.

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The results obtained with NDV, replacing another paramyxovirus (Sendai virus) in the assay, were satisfactory, and repeatability was shown. It was soon demonstrated that a substance which behaved as an interferon appeared in the serum of cattle during illness with ephemeral fever. The laboratory controls, serum with a high titre of interferon and one with no detectable effect on the assay system, demonstrated that there was minor variation between tests carried out on different days. When the series of samples from a single cow were repeated in tests carried out on different days the actual titres did vary.

However, the general pattern did not. NDV does not naturally infect cattle so there is no possibility that antibody to NDV was mimicking the effect of interferon. Also, serial serum samples from each animal, some before inoculation, provided a control against this possibility. It was demonstrated by the use of serial serum samples from every clinical case of ephemeral fever that the activity interfering with the NDV replication was transitory and preceded the development of specific antibodies.

In natural cases of ephemeral fever, interferon appeared in the serum before the development of the obvious clinical signs. The time relationship could not be determined with the same precision as in experimental cases because blood samples were collected only once or twice a day. Nevertheless the interferon levels attained were of similar magnitude in the natural and experimental cases. Again, the serial samples available from each cow showed that interference with NDV replication developed before clinical signs became obvious.

The limitation of the sensitivity of the method used meant that interferon could not be detected in undiluted serum. Thus it was not possible to determine the precise time at which interferon appeared at levels less than 1:8 in the serum. This same limitation was found with assays of interferon in human serum (G.M. Scott, personal communication). The antiviral activity of the serum was contained in the fractions with a molecular size less than that of albumen. This is well below the size of the globulin fraction, and is direct evidence that the antiviral activity of the serum was not due to antibodies.

The interferon in the serum of cattle with ephemeral fever is twice as labile at pH 2 than at pH 4. However, even overnight treatment (19h 20 min) with low pH did not produce a greater effect than treatment for 2 h. In contrast 95% of activity was removed by exposure to 56°C for 2 h. This is a similar result to that of Letchworth and Carmichael (1983). Stewart II (1981) has thrown doubt on the value of pH and heat stability in classifying interferons as Class I and Class II. The results illustrated in Figure 2 are consistent with a mixture of pH stable and labile interferons being present.

High interferon levels were demonstrated in cattle experimentally infected with BEF virus, the rapid rise in interferon levels preceding the fever, (Table 1) and the fall occurring as fever subsided. Besides having an important role in checking the multiplication of BEF virus before antibody circulates, it is possible that the high levels of interferon may be inducing toxic effects. However, interferon was consistently detected and rose to high levels in the serum before the appearance of clinical signs. It can be concluded interferon is a potential cause of the clinical effect(s) as its rise precedes the onset of illness. The statistical correlation does not prove cause and effect but suggests interferon rise and subsequent fever are linked.

The toxic effects of a single dose of human leucocyte interferon on human volunteers was described by Scott *et al.* (1981). The major clinical effects were fever, headache, malaise, chills, fatigue, nausea and anorexia. There is a

marked fall in the number of lymphocytes to about 25% of normal and a sharp rise in the neutrophil numbers. There are close similarities between these effects in the human volunteers and the earlier phase of ephemeral fever in cattle.

Young (1979) described the production of interferon in cattle experimentally infected with BEF virus, using an assay system which measured plaque reduction in bovine fibroblasts infected with a bovine picornavirus designated as 66/27. He found peak titres which varied from 80 to 160. However, these titres cannot be compared with those obtained by other methods as the relative sensitivity of the various assay systems is unknown. The time relationship between illness and interferon appearance in Young's series is not clearly expressed. He drew no conclusion as to the role of interferon, but tended to agree with Fenner (1974), that it was a factor in recovery from disease. A role for interferon limiting BEF virus replication is probable, as recovery from ephemeral fever is complete in experimentally infected cattle 72-96 hours before neutralizing antibodies are detected. The natural cases were complicated by the presence of neutralizing antibodies generated by heterotypic infections before the onset of ephemeral fever (St.George *et al.* 1984) which result in an anamnestic antibody response. Thus the same time lag between recovery and the detection of neutralizing antibodies does not occur, a rise in the titre of neutralizing antibody beginning in natural infections prior to complete recovery.

If interferon is a critical element in a series of biochemical events leading to the induction of the severe clinical effects which constitute ephemermal fever, rather than cytopathological effects of BEF virus in muscles and joints, important supporting evidence can be provided by specific treatment. In the studies by Scott *et al.* (1981) and G.M. Scott (personal communication), indomethacin effectively reduced the severity of the toxic reactions of synthetic interferons. There has been anecdotal evidence for many years that anti-inflammatory drugs have beneficial effects on cattle with ephemeral fever and some field evidence of this was observed by St.George *et al.* (1984).

"Since the experiments reported here were completed, the effects of pretreatment of cattle with phenylbutazone have shown that the fever and other clinical signs associated with experimental ephemeral fever can be prevented (St. George *et al.* 1986; Uren *et al.* 1989). This lends support to the contention that BEF virus causes disease indirectly through a mediator. Interferon is thus a

candidate. However, levels of interferon are not altered by phenylbutazone treatment (St. George *et al.* 1986). The effect must be on a biochemical mediator between interferon and target cells."

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