FATE OF INTRAVENOUSLY ADMINISTERED INTERFERON AND THE DISTRIBUTION OF INTERFERON DURING VIRUS INFECTIONS IN MICE

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THERE have been a number of reports on interferon production by animals during the course of virus infections. Certain important questions must, however, be answered before blood and tissue interferon titres can be properly evaluated. For instance, it is necessary to know whether interferon stays in the blood or whether it is removed by organs, and if so, by which organs and at what rate.

In the present report, an attempt has been made to provide the background for interpreting blood and tissue interferon titres by following the fate of intravenously injected interferon. Studies have also been made of the effects of various factors involved in processing and assay on interferon titres. Following this is a survey of the distribution of interferon in mice during infection with West Nile, ectromelia and influenza viruses. The findings are discussed in the light of the results of the first part of the study and the main conclusion, that the spleen plays an important part in interferon production under certain circumstances, has been confirmed by further experiments.

MATERIALS AND METHODS

Mice

Multicoloured, outbred mice of the Walter and Eliza Hall Institute strain were used at 6–8 weeks of age.

Viruses

Ectromelia virus.—The 2 strains of ectromelia virus used have been described by Fenner (1949). The Hampstead mouse strain derives by mouse passage only, from the original virulent strain isolated by Marchal (1930). A first egg passage material was used.

The Hampstead egg strain originated from a Hampstead mouse liver preparation which had been passed about 60 times in eggs when used by Fenner (1949) and an unknown number of times since then. It is much less virulent than the Hampstead mouse strain.

West Nile virus.—A mouse brain preparation of the Sarafend strain, passed 6 times in mouse brains since isolation, was used.

Influenza virus.—Infected allantoic fluid containing the PR8 strain of influenza virus was used.

Semliki forest virus.—A strain of virus obtained from the Rockefeller Foundation Virus Laboratory and passed 20 times in mouse brain and once in chick embryo fibroblasts since isolation, was used. Stock virus was contained in the medium from infected chick embryo fibroblast cultures.

Mouse embryo fibroblast cultures

Confluent secondary monolayers were made from 15–16-day-old mouse embryos. The growth medium consisted of Eagle's medium containing 7 per cent heated calf serum (56° for 30 min.). The cultures were overlaid after infection with Earle's saline containing 0·5 per

cent lactalbumin hydrolysate, 0·1 per cent yeast extract, 0·1 per cent bovine plasma albumin fraction V, 2 per cent heated calf serum, 0·01 per cent DEAE dextran and 0·75 per cent Difco agar.

Interferon assay

Except when otherwise stated, all materials for assay were dialysed at 4° against Glycine-HCl buffer (pH 2·0) overnight and returned to pH 7·2 by further dialysis at 4° against several changes of Dulbecco's phosphate buffered saline. The dialysed material was centrifuged at 100,000 *g* for at least 60 min. in a Spinco model L centrifuge and the supernatant fluid tested for interferon activity.

In the assay, mouse embryo fibroblast monolayers were treated for 5 hr. at 37° with 5 ml. of the appropriate dilution of the material in Eagle's medium. The sensitivity of the method could not be improved by employing smaller volumes of fluid or longer periods of adsorption. The interferon containing medium was then completely removed and the monolayers infected with approximately 50 pfu of Semliki Forest virus in 0·1 ml. medium. After adsorption for 75 min. at 37°, overlay medium was added. Plaque counts were made on the second day, after staining the plates with 1 : 20,000 neutral red for 2–3 hr. at 37°. At least 5 plates were used for each dilution of the interferon preparation.

One unit of interferon is defined as the amount, contained in 5 ml., which reduces the plaque count by 50 per cent (PDD_{50}). This was estimated on each occasion from a graph obtained by plotting the logarithm of the reciprocal of the dilution against the response expressed as the percentage of the control plaque count. At least three suitably spaced dilutions were included in each estimation.

The stock interferon preparation maintained at −60° was assayed on each occasion, as a guide to the reproducibility of the method. A single stock of interferon preparation was found to vary as much as 4-fold in titre, in assays on different occasions under similar conditions. This is similar to the results reported by Lindenmann and Gifford (1963) in the case of the titration of chick interferon.

Stock interferon

A stock of interferon was prepared from the brains of mice infected with about 600 LD_{50} of West Nile virus and killed on the fourth day, when the brains are known to contain large amounts of interferon (Finter, 1964). This stock of interferon with a titre of 800 units per ml. was stored in aliquots at −60°.

RESULTS

Preliminary experiments

Absence of interferon-like inhibitors in blood and tissues.—There was no interferon-like activity in 1 : 8 dilutions of the blood or 1 : 2 dilutions of the 10 per cent suspensions of the liver and spleen of normal mice.

Absence of effect of normal blood and tissues on interferon activity.—Stock interferon was mixed with normal mouse serum and with heparinised fresh normal mouse blood. In another test, blood from normal mice was allowed to clot in the presence of stock interferon. In a third experiment, livers, spleens, kidneys and brains from normal mice were ground in a chilled mortar and pestle with stock interferon and processed. The treatments did not have any significant effect on the interferon titre.

Effect of the method of preparation on interferon titres.—A West Nile virus infected mouse brain suspension was divided into two portions. One portion was processed as usual, while the other one was only centrifuged at 100,000 × *g* for 90 min. to remove the virus. Both preparations gave identical titres of 800 units per ml. In another experiment it was found that when stock interferon was reprocessed, its titre remained unchanged. Thus the treatments involved in preparing material for interferon assay do not affect the interferon titres.

Thermostability of mouse interferon.—The stability of mouse interferon under conditions of storage (−60°) and at body temperature (37°) was investigated. Stock interferon, maintained at −60°, was thawed and titrated at various times after preparation. Comparison of the titres obtained in experiments using challenges in the range of 50 to 100 pfu of Semliki Forest virus (Table I) shows that mouse interferon, stored at −60°, does not suffer any loss in titre for at least 146 days.

TABLE I.—*Stability of Interferon Stored at* −60°

Interval between preparation and testing (days)	3	39	65	146
Titre (units/ml.)	800	500	2000	1600

The stability of interferon at body temperature (37°) was studied in two experiments. In the first, stock interferon was mixed with 2 volumes of fresh normal mouse serum and incubated at 37°. At the start of the experiment, the titre was 320 units per ml. and this fell to 160 units per ml. by 7 hr and to 40 units per ml. by 20 hr, showing that under these conditions interferon has a half life of about 6 hr. In the second experiment, stock interferon was mixed with 2 volumes of heparinised fresh normal mouse blood and incubated at 37°. The initial titre was 500 units per ml. It was unchanged at 3 and 6 hr. and had dropped to 400 units per ml. by 18 hr. Thus interferon is fairly stable in normal mouse blood.

Fate of intravenously administered interferon

In order to interpret blood interferon levels and to understand the possible spread of interferon to organs, information regarding the fate of interferon injected into blood was needed.

Therefore, 340 units of interferon were inoculated intravenously to each of 6 mice (experiment 1, Table II). Three of the mice were killed at 5 min. and the

TABLE II.—*Recovery of Interferon from Organs of Mice after Intravenous Administration*

	Experiment 1 (interferon input 340 units/mouse) Interferon recovery (units/organ) at		Experiment 2 (interferon input 150 units/mouse) Interferon recovery (units/organs) at
	5 min.	6 hr.	5–13 min.
Blood*	28	<7	20
Brain	<4	<3·6	<0·8
	(0·16)	(<0·04)	(0·1)
Kidney	2·4	<7·2	20
	(1·8)	(<0·45)	(1·3)
Liver	<21†	<21	21
	(6·5)	(<1·6)	(4·7)
Lung	1·65	<3	4·2
	(0·1)	(<0·025)	(0·075)
Spleen	<3·4	<3·4	<3·4
	(0·14)	(<0·035)	(0·1)

Figures in brackets represent the possible maximum contribution from the blood content of the organ (calculated on the basis of the figures given by Kaliss and Pressman, 1950).

* Calculated from serum or plasma titrations.

† Plaque size but not number reduced at the lowest dilution tested.

other 3 at 6 hr. Blood, brain, kidney, liver, lung and spleen were collected and stored at −60° within 30 min. of collection and until processed. The results of interferon titrations on these materials show that the injected interferon disappears rapidly from circulation. Only very small amounts were, however, detectable in the organs at 5 min., while the 6 hr. specimens of blood and organs contained no detectable interferon. It was thought possible that larger amounts of interferon could have been present in the organs when the mice were killed, but had been rendered undetectable during the 30 min. when the organs were held at room temperature prior to storing at −60°. This was shown to be the case in an experiment where 160 units of interferon were injected intravenously to each of 3 mice and their livers harvested 5 min. later. Portions of each liver which were snap-frozen immediately and pooled gave an interferon titre of 25 units per g., whereas the remaining portions of the liver, when snap-frozen after 30 min. at room temperature contained only 4 units per g.

Two further experiments were then carried out to study the distribution of intravenously injected interferon. In the first experiment, 10 mice were each injected intravenously with 150 units of interferon, and killed at 5 min. The blood was collected directly from the heart with a heparinised syringe within 2 min. of death and stored in an ice bath until the plasma was separated 30 min. later. The brain, kidney, liver, lung and spleen were collected and snap-frozen in liquid nitrogen cooled tubes within 8 min. of death. They were stored at −60° until processed. A tube of stock interferon held under the same conditions as the blood and stored at −60° at the same time as the plasma, served as the control. The results of interferon assay on the materials again show that the injected interferon rapidly disappears from blood, but approximately 40 per cent of it was detected in the blood, kidney, lung and liver. None was detected in the brain or spleen. In a subsequent experiment, these findings were confirmed and it was also found that neither skeletal nor cardiac muscle contain detectable interferon 5 min. after intravenous administration of interferon. It is clear, therefore, that intravenously injected interferon is rapidly removed from the blood and is to a significant extent recoverable in the tissues, if these are snap-frozen within 13 min. of the injection.

The interferon, used in the clearance experiments described so far, was prepared by routine extraction from mouse brain. It was thought possible that the interferon occurring naturally in mouse plasma might be dealt with in a different way. One ml. of unprocessed plasma interferon, obtained from 20 inbred Bagg mice $4\frac{1}{2}$ hr. after a large dose of intravenous influenza virus, was inoculated intravenously to each of 3 normal Bagg mice. Each mouse received 80 units of interferon. One mouse was killed at 5 min. and 2 at 1 hr. The serum of the mouse killed at 5 min. contained 20 units of interferon, but the amount in the serum at 60 min. was too low for detection. Thus, there was no essential difference in the clearance of processed or native interferon.

Distribution of interferon in virus infected mice

West Nile, ectromelia and influenza viruses were used. West Nile virus was chosen because it is neurotropic in mice and because very large amounts of interferon appear in the infected brains (Finter, 1964). Ectromelia virus infection in mice leads to a generalised disease, the pathogenesis of which has been worked out

in detail (Fenner, 1948; Mims, 1959; Roberts, 1963). Influenza virus, given intravenously in large amounts, was found to cause very rapid appearance of circulating interferon in the same manner as Newcastle disease virus (Baron and Buckler, 1963).

West Nile virus.—Mice were injected intracerebrally with 600 LD_{50} of West Nile virus and killed on the 4th day, and blood, brain, kidneys, liver, lung and spleen were collected. The serum and 10 per cent suspensions of the organs were titrated for virus on chick embryo fibroblast monolayers and after processing, for interferon.

TABLE III.—*Distribution of Interferon during West Nile Virus Infection of Mice*

	Blood*	Brain	Kidney	Liver	Lung	Spleen
Virus content (log pfu)	1·8	9·7	1·9	—	1·6	<0·7
Interferon (units) content	14	680	<3·7	2·6	<8·5	<17
		(0·07)	(0·9)	(3·3)	(0·05)	(0·06)
Concentration†	7	1700	<10	2	<57	<200

* Interferon present in the total blood volume (2 ml./mouse), calculated from the serum titre. Interferon contents of organs are calculated from the titres of 10 per cent suspensions.
† Interferon concentration expressed as units per ml. or as units per g. of wet weight of tissue. Figures in brackets give the possible maximum contribution from the blood content of the organ.

The results (Table III) show that the brains of these animals contained large amounts both of interferon and virus. Interferon was not detected in the lung, kidney or spleen and in these organs, there were only small amounts of virus. The interferon content of the liver could be accounted for by the blood present in it. Clearly, under these circumstances West Nile virus does not grow to a significant extent or cause interferon production in organs other than the brain. Virus titres were also very low in the blood but moderate amounts of interferon were, nevertheless, present suggesting that some of the interferon produced in the brain spills over into the blood.

Ectromelia virus.—Foot pad infection: Mice were injected with $10^{4.7}$ pock-forming units of Hampstead egg (attenuated) virus into the footpad. They were killed on the sixth day and the serum and the organs titrated for interferon. The largest amounts of interferon were present in the spleen (Table IV). It was also found in significant amounts in the serum, liver and lymph nodes, but not in the inoculated footpad.

Intravenous infection: Mice were injected intravenously with 10^7 pock-forming units of either the virulent (Hampstead mouse) or the attenuated (Hampstead egg) strain and killed 4, 12 and 48 hr. later. The serum and organs were collected and assayed for interferon activity. The mice given the virulent strain were very sick at 48 hr.

The results (Table IV) show that interferon was present in the serum of only the mice given the virulent virus but not until 48 hr. after infection, when the animals were sick. Interferon was not detected in the livers at any time. The spleen contained small amounts of interferon at 48 hr., the titres in both groups of mice being identical.

Thus, the organs of the mice 2 days after intravenous injection of virus had less interferon than 6 days after subcutaneous injection, even though the intravenous infection was with virulent virus and the mice were sick at 48 hr.

TABLE IV.—*Distribution of Interferon During Ectromelia Virus Infection of Mice*

Interferon (units) in	Hampstead mouse (virulent) intravenous 4 hr.	12 hr.	48 hr.	Hampstead egg (attenuated) intravenous 4 hr.	12 hr.	48 hr.	footpad 6 days
Blood* Content	<1·5	<7·5	7·5	<37·5	<7·5	<7·5	25
Concentration†	<0·75	<3·8	3·8	<18·8	<3·8	<3·8	12·5
Footpad Content	—	—	—	—	—	—	<5
Concentration†	—	—	—	—	—	—	
Liver Content	<26	<26	<26	<26	<26	<26	81·9 (5·9)
Concentration†	<20	<20	<20	<20	<20	<20	63
Lymph nodes Content	—	—	—	—	—	—	22
Concentration†	—	—	—	—	—	—	1000
Spleen Content	<1·7	<1·7	5·1 (0·04)	<1·7	<1·7	5·1 (<0·04)	100 (0·1)
Concentration†	<20	<20	60	<20	<20	60	1170

* Interferon present in the total blood volume (2 ml./mouse), calculated from the serum titre. Inteferon contents of organs are calculated from the tires of 10 per cent suspensions.
† Interferon concentration expressed as units per ml. or as units per g. of wet weight of tissue. Figures in brackets give the possible maximum contribution from the blood content of the organ.

Influenza virus.—Two mice were each inoculated intravenously with about 10^3 HAU of influenza virus and killed after $4\frac{1}{2}$ hr. The blood was collected and kept in an ice bath until serum was separated and stored at −60°. Brain, kidney, liver, lung, muscle and spleen were collected in liquid nitrogen and stored at −60°. All the materials were assayed for interferon activity.

TABLE V.—*Distribution of Interferon During Influenza Infection of Mice*

	Blood*	Brain	Kidney	Liver	Lung	Muscle	Spleen
Content (units)	250	<5 (1·3)	1·6 (16·3)	21 (58·8)	2·7 (0·9)	0	2·6 (1·3)
Concentration†	125	<12·5	4	16	18	0	31

* Interferon present in the total blood volume (2 ml./mouse), calculated from the serum titre. Interferon contents of organs are calculated from the titres of 10 per cent suspensions.
† Interferon concentration expressed as units per ml. or as units per g. of wet weight of tissue. Figures in brackets give the possible maximum contribution from the blood content of the organ.

The results (Table V) show that the blood contains large amounts of interferon in contrast to the small amounts present in the organs. The interferon content of all organs, except perhaps the spleen, can be accounted for either by their blood content or on the basis of interferon uptake from the blood. The results were taken to indicate that the spleen probably produces interferon under these circumstances.

Production of interferon by the spleen

The distribution of interferon in mice infected with ectromelia virus and in those given an intravenous injection of influenza virus suggested that the spleen might play an important part in the production of interferon. The role of the spleen in the quick interferon response to influenza virus was investigated in an experiment with splenectomised animals.

Two mice were splenectomised and 3 days later, were injected intravenously with approximately 10^3 HAU of influenza virus. They were killed $4\frac{1}{2}$ hr. after injection of the virus and the sera pooled. The pooled serum contained only one sixth as much interferon as pooled serum from normal mice given the same dose of the virus (Table VI). In a similar experiment, mice were found to have an impaired interferon response when tested one month after splenectomy (Table VI).

TABLE VI.—*Effect of Splenectomy on Interferon Production*

	Serum interferon titre (units/ml.)	
	Expt. 1	Expt. 2
Normal control	200	126
3 days after splenectomy	32	—
1 month after splenectomy	—	11

It is, therefore, clear that the spleen plays an important role in interferon production following the intravenous injection of influenza virus.

DISCUSSION

The results presented in this paper permit some general conclusions. Firstly, interferon is unchanged in titre during grinding up and processing with normal organs and tissues. But, interferon present in freshly removed tissues may become undetectable unless the tissues are snap-frozen without delay. Secondly, interferon in normal mouse blood at body temperature is completely stable for at least 6 hr. and remains largely unaffected for 18 hr. or longer. Thirdly, interferon disappears rapidly from the blood. Therefore, interferon assays of organs as routinely collected may considerably underestimate the amounts originally present and the detection of appreciable amounts of interferon in the blood implies a very rapid release of large amounts into the blood. Furthermore, the absence of detectable interferon in the blood does not rule out the possibility that it is being released into the blood. The rapid clearance of interferon from blood may help to explain the difficulty in finding interferon in sera collected during clinical viral infections (Wheelock and Sibley, 1964). While this report was being prepared, Baron, Buckler, McCloskey and Kirschstein (personal communication) reported that intravenously injected interferon is cleared from blood to the extent of 86 per cent in 30 min. and more than 97 per cent in 1 hr. Finter (personal communication) has more recently reported similar findings.

The experiments reported here also show that 5 min. after an intravenous injection of interferon when most of it has left the blood, a significant proportion of it can still be detected in the organs. The lung and kidney accounted for nearly one-sixth of the injected interferon. In contrast, the spleen and the liver, organs rich in reticuloendothelial cells, contained little or none. The latter organs, therefore, either take up interferon less avidly or else take it up avidly and then rapidly render it undetectable. The brain, which is a very rich source of interferon when it is infected with some viruses, contained none of the intravenously injected interferon. Muscles, which have large capillary beds like the kidney and the lung, also did not contain any of the injected interferon.

In mice infected with West Nile virus, interferon titres in the brain paralleled virus titres, whereas all the other organs tested had no interferon and very little virus. Interferon, therefore, seems to be produced only in the significantly infected organ. The presence of appreciable amounts of interferon in the blood is taken as evidence for interferon spill over from brain. This overflow has to be substantial to maintain the observed titres in blood, in view of its rapid clearance.

In mice infected with ectromelia virus, the spleen contained the highest concentration of interferon in each experiment. When tested 6 days after footpad inoculation of the attenuated virus, for instance, the interferon concentration in the spleen of mice was about 20 times that in the liver. These findings strongly suggest interferon production by the spleen in mice infected with ectromelia virus, especially if it is also remembered that intravenously injected interferon is not detected in the spleen. The lymph nodes also contained high concentrations of interferon. Probably the high interferon concentrations found in the spleens and lymph nodes of ectromelia infected mice merely reflect the growth of virus in these tissues, just as the high interferon titres in the brains of West Nile infected mice reflect the growth of virus in the brain.

The association between high virus titres and the presence of significant amounts of interferon in organs has also been observed by other workers (Vainio, Gwatkin and Koprowski, 1961; Vilcek, 1964; Vilcek and Stancek, 1963; Heineberg, Gold and Robbins, 1964) though a strict parallelism is not always seen (Baron, du Buy, Buckler and Johnson, 1964). However, the growth of virus in an organ is not by itself enough to cause interferon production in the organ, as is seen in the case of the livers of mice injected intravenously with ectromelia virus. The liver is the major target organ involved in ectromelia virus infection, but interferon could not be detected in the liver even at a time when it is known to contain large amounts of virus, and Kupffer and hepatic cells are infected on a large scale (Mims, 1959). On the other hand, there were large amounts of interferon in the liver 6 days after subcutaneous infection with an attenuated strain, and it is known that there would have been considerably less growth of the virus in the liver under such circumstances.

After intravenous injection of 10^7 pfu of the virulent strain of ectromelia virus, there was less than 7·5 units/ml. of interferon in the serum at 4 hr. It is interesting to note that Baron and Buckler (1963) also found only 3 units/ml. of interferon in the serum of mice $4\frac{1}{2}$ hr. after the intravenous injection of $10^{5.9}$ pfu of a strain of vaccinia virus. In contrast to these results, very large amounts of circulating interferon were produced after the intravenous injection of influenza virus, but the 1000 HAU of influenza virus which were injected would have contained about 10^{10} virus particles (Fazekas de St. Groth and Webster, 1963), in comparison with a maximum of 10^8 particles in the case of the poxvirus injections.

Although large amounts of interferon are present in the blood $4\frac{1}{2}$ hr. after a large intravenous dose of influenza virus, only small amounts are present in the organs, and except perhaps in the case of spleen, the interferon present can be either attributed to the blood content, or to the uptake from the blood by these organs. Removal of the spleen considerably impaired the interferon response of the mouse to intravenously injected influenza virus, providing direct evidence for the important part played by the spleen in interferon production following the intravenous injection of influenza virus. Kono and Ho (1965) found that in rabbits given an intravenous injection of a large dose of Sindbis virus, interferon

activity was highest in the spleen and blood, and cell suspensions from the spleen were capable of quick and sustained interferon production *in vitro*. Heineberg *et al.* (1964) reported that in adult mice infected with Coxsackie B virus, high titres of interferon and virus are quickly attained in the spleen.

SUMMARY

Normal mouse serum and tissue extracts did not contain any interferon-like agent nor did they inactivate interferon.

Mouse interferon was stable for at least 5 months at −60°, and it had a half life of more than 18 hr. in normal mouse blood at 37°.

Intravenously injected mouse interferon disappeared rapidly from the blood but a significant proportion of it could be detected in the liver, lung and kidney 5 min. after injection, if the organs were frozen immediately. None could be detected in the brain or spleen.

Mice infected by the intracerebral inoculation of West Nile virus had high titres of both virus and interferon in the brain and very little virus or interferon in other organs. During ectromelia infection, interferon was consistently detected in the spleen in high concentrations.

Large amounts of interferon were present in the serum 4½ hr. after the intravenous injection of influenza virus, but interferon production was considerably reduced by splenectomy.

REFERENCES

Baron, S. and Buckler, C. E.—(1963) *Science*, **141**, 1061.
Baron, S., du Buy, H. G., Buckler, C. E. and Johnson, M. L.—(1964) *Proc. Soc. exp. Biol. Med.*, **117**, 338.
Fazekas de St. Groth, S. and Webster, R. G.—(1963) *J. Immun.*, **90**, 151.
Fenner, F.—(1948) *J. Path. Bact.*, **60**, 529—(1949) *Aust. J. exp. Biol. med. Sci.*, **27**, 31.
Finter, N. B.—(1964) *Nature, Lond.*, **204**, 1114.
Heineberg, H., Gold, E. and Robbins, F. C.—(1964) *Proc. Soc. exp. Biol. Med.*, **115**, 947.
Kaliss, N. and Pressman, I. D.—(1950) *Proc. Soc. exp. Biol., Med.* **75**, 16.
Kono, Y. and Ho, M.—(1965) *Virology*, **25**, 162.
Lindenmann, J. and Gifford, G. E.—(1963) *Virology*, **19**, 302.
Marchal, J.—(1930) *J. Path. Bact.*, **33**, 713.
Mims, C. A.—(1959) *Br. J. exp. Path.*, **40**, 543.
Roberts, J. A.—(1963) *Br. J. exp. Path.*, **44**, 465.
Vainio, T., Gwatkin, R. and Koprowski, H.—(1961) *Virology*, **14**, 385.
Vilcek, J.—(1964) *virology*, **22**, 651.
Vilcek, J. and Stancek, D.—(1963) *Acta virol.*, **7**, 331.
Wheelock, E. F. and Sibley, W. A.—(1964) *Lancet*, ii, 382.