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Hyporeactivity to Interferon Induction: Characterization of a Hyporeactive Factor in the Serum of Encephalomyocarditis Virus-Infected Mice

DALE A. STRINGFELLOW

Experimental Biology, The Upjohn Company, Kalamazoo, Michigan 49001

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Mice infected with encephalomyocarditis virus develop a severe state of hyporeactivity to interferon induction. One mechanism possibly responsible for development of hyporesponsiveness in these animals is a circulating factor which can be detected in their serum 96 h after encephalomyocarditis virus infection (at the time of peak hyporeactivity in vivo). This report describes some of the physicochemical characteristics of this serum hyporeactive factor (SHF). SHF is a protein with a molecular weight between 20,000 and 40,000 that was extremely labile at 56 C, losing greater than 90% of its biological activity in 8 min, but stable at 37 C for at least 4 h. Hyporeactive factor was also stable over a pH range of 2 to 11 for 48 h at 4 C. These results suggest that SHF is physicochemically similar to interferon. However, no interferon could be detected in the SHF preparation, and no loss in biological activity was observed when the serum factor was incubated with anti-interferon antibody, suggesting that they are separate substances.

Mice infected with encephalomyocarditis (EMC) virus progressively lose their ability to produce interferon upon exposure to any of several viral or nonviral inducers (11). Although an explanation for the progressive decrease in interferon responsiveness of EMC virus-infected mice is not available at the present time, Stringfellow and Glasgow (12) have reported that serum taken from mice 96 to 120 h after infection with EMC virus does contain a factor(s) capable of transferring hyporeactivity to otherwise normal murine cells in vitro. These findings were particularly interesting since neither interferon nor EMC virus could be detected in the hyporeactive serum and suggested that another factor (serum hyporeactive factor [SHF]) may be responsible for the development of hyporesponsiveness in infected animals.

Mice infected with other viruses (Friend leukemia virus [3], cytomegalovirus [8], and lymphocytic choriomeningitis virus [5]) also develop a state of hyporeactivity. DeMaeyer-Guignard (3) has reported that serum from Friend leukemia virus-infected mice may also contain a factor capable of mediating hyporeactivity. If a similar state of hyporesponsiveness develops in man as a consequence of infection, it may represent a major limitation to the use of interferon inducers as effective therapeutic, antiviral agents. The following studies were, therefore, undertaken to determine the physicochemical nature of SHF and in so doing add further insight into how or why animals develop hyporeactivity. Such information would seem essential if means of overcoming the limitations associated with such a condition are to be developed.

MATERIALS AND METHODS

Mice. Female ICR strain mice (Upj:tuc-ICR) were provided by Upjohn Rearing and Procurement and were housed under conditions of constant temperature and a 12-h light cycle, with food and water provided ad libitum. After being received from the breeder, mice were housed as described for at least 1 week to allow them to adjust to the new environment before being used.

Cells. Murine interferon assays were carried out in a cloned continuous line of mouse L-cell fibroblasts (L_{929}) originally obtained from the American Type Culture Collection Cell Repository. Secondary mouse embryo fibroblasts (MEF) were obtained by sacrificing pregnant ICR mice near term. The embryos were removed aseptically and washed in phosphate-buffered saline (PBS), and the head and limbs were removed. The embryos were then minced, washed in PBS, and trypsinized. After centrifugation, cells were resuspended in Eagle minimum essential medium (MEM) at 5×10^4 cells/ml and distributed into sterile 32-oz (ca. 0.95-liter) prescription bottles, 100 ml/bottle. After incubation for 3 days at 37 C, confluent monolayers were obtained. These cells were removed

from the glass bottles with 0.25% trypsin (1:250, Difco) and suspended at 5 × 10^5 cells/ml in MEM. Two milliliters of this preparation was distributed into each 35-mm plastic petri dish. Confluent monolayers were obtained in 48 h.

Media. Eagle MEM (Microbiological Associates) containing 10% fetal calf serum (Reheis Chemical Co., Phoenix, Ariz.), 100 U of penicillin per ml, and 50 μg of streptomycin per ml was used to propagate and maintain all tissue cultures.

Viruses. The Herts strain of Newcastle disease virus (NDV) was originally obtained from S. Baron (National Institutes of Health [NIH]). The stock NDV preparation used in these experiments was propagated in embryonated hen eggs which had been injected by the allantoic route and had a titer of 2.0 × 10^9 plaque-forming units (PFU)/ml in primary chicken embryo cells.

Vesicular stomatitis virus (VSV), Indiana strain, was obtained from the American Type Culture Collection (Rockville, Md.). Pools of VSV were propagated in primary chicken embryo cell monolayers. The stock preparation of VSV used in these studies had a titer of 2 × 10^7 PFU/ml when assayed in L_{929} mouse cells.

The strain of EMC virus used in these studies was a large plaque variant originally obtained from K. K. Takemoto (NIH). Suckling mice were injected by the intracerebral route, and brains were removed at the height of disease. These tissues were homogenized, and a 10% suspension in MEM was used as a stock pool with a titer of 2.5 × 10^8 PFU/ml in L_{929} mouse cells.

Interferon assay. Confluent monolayers of L_{929} cells grown in 35-mm plastic petri dishes (Falcon Plastics) were treated with 1 ml of an appropriate interferon dilution overnight at 37 C. The plaque reduction assay used VSV as the challenge virus and has been previously described (11). An internal laboratory reference murine interferon standard was included with each interferon assay, and the sensitivity of our assay was compared with the International Murine Standard obtained from the NIH. In our system, the International Standard had a mean titer of approximately 4,200 U/ml compared with its accepted titer of 6,500 U/ml.

Preparation of serum from EMC virus-infected mice. Female ICR strain mice (20 g) were infected intraperitoneally with a 100% lethal inoculum (1,000 PFU) of EMC virus. One hundred hours after infection, mice were bled by cardiac puncture. The blood was allowed to clot at room temperature for 1 h, and the clot was then rimmed and refrigerated at 4 C overnight. Serum was collected and assayed for interferon and EMC virus on L_{929} cells. The pool of SHF used in these experiments was found to contain no virus or interferon at a 1:10 dilution but could, when diluted as high as 1:200, inhibit by 50% or more the interferon response induced in secondary MEF cells by NDV.

Mice from the same animal shipment which were not infected with EMC virus were bled, and their serum served as the normal mouse serum control throughout these studies.

Hyporeactivity assay. Serum to be assayed for its ability to transfer hyporeactivity to normal cells was diluted serially in MEM. Growth medium was removed from confluent 2° MEF monolayers grown in 35-mm plastic petri dishes (Falcon Plastics), and plates (in triplicate) received 1 ml of the appropriate dilution of serum from EMC virus-infected mice, normal mouse serum, or MEM. Cell cultures were returned to 37 C for 24 h, at which time each plate received 0.1 ml of NDV (4 × 10^7 PFU) or MEM. Growth medium was harvested from cells 24 h after inducer challenge and frozen at −20 C until assayed for interferon. All samples were held at a pH of 2.0 for 5 days at 4 C before being assayed for interferon.

RESULTS

The following studies were undertaken to determine the physical-chemical nature of the hyporeactive factor in serum from EMC virus-infected mice (SHF). In each case the hyporeactive assay and interferon assays were run as described in Materials and Methods. A single pool of serum from normal or EMC virus-infected mice was used throughout these studies.

Heat stability of SHF. The stability of SHF was determined at 56 and 37 C. Serum from EMC virus-infected or normal uninfected mice was diluted 1:5 in MEM and divided into 0.75-ml portions. At 0 time all tubes were immersed in a 56 or 37 C water bath. At specified times tubes were removed and immediately frozen in a dry ice-acetone bath. All samples were stored frozen at −20 C until assayed for their ability to reduce the interferon response of 2° MEF cells to NDV. The results illustrated in Fig. 1 indicate that at 56 C SHF was extremely labile. By 8 min, greater than 90% of the biological activity of SHF was lost. For example, cells treated with a 1:30 dilution of unheated serum from infected mice produced only 270 U of interferon per ml of growth media, whereas cells treated with the same dilution of serum heated at 56 C for 8 min produced 1,700 U of interferon per ml. No appreciable difference was seen between SHF heated at 56 C for 8 min or longer or normal mouse serum. Apparently almost all activity was destroyed within the first 8 min. The interferon response of cells treated with each dilution of serum heated at 56 C for 4 min consistently produced more interferon than did cells treated with comparable dilutions of unheated serum, indicating that some biological activity was lost within 4 min.

Figure 2 illustrates the stability of SHF at 37 C. The results indicate that there was no appreciable loss of biological activity within 4 h at 37 C. Cells treated with each dilution of serum held at 37 C for 1, 2, or 4 h produced approximately the same amount of interferon in

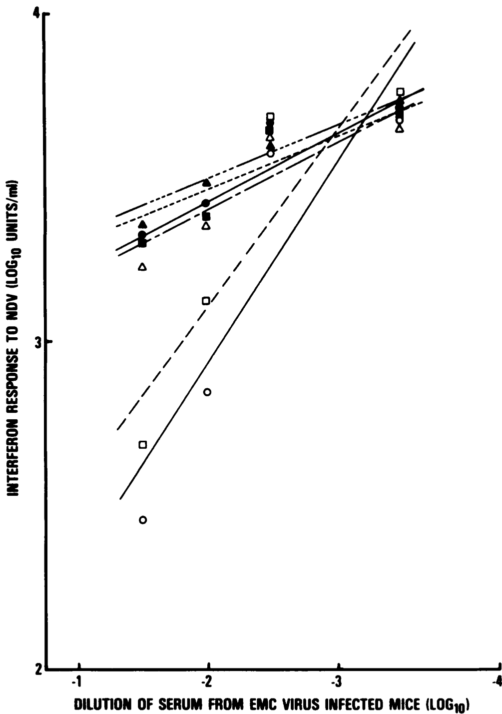

FIG. 1. *Instability of SHF at 56 C. Interferon response induced by NDV in 2° MEF cells treated with serum from normal or EMC virus-infected mice (SHF). Symbols:* O, *Unheated SHF control; SHF held at 56 C for:* □, *4 min;* Δ, *8 min;* ●, *15 min;* ▲, *30 min; and* ■, *unheated normal mouse serum.*

response to NDV as cells treated with comparable dilutions of serum from EMC virus-infected mice that was not heated.

In both Fig. 1 and 2, as well as each subsequent figure, the line describing each set of data points represents the line of best fit, estimated by using a linear regression analysis of variance.

Dialysis of SHF. One means of roughly estimating the molecular size of the hyporeactive factor in serum from infected mice was to determine if it could pass through dialysis tubing. To investigate this, 2-ml samples of serum from normal or EMC virus-infected mice were placed into separate dialysis bags made from dialysis tubing that would restrict the migration of molecules with a molecular weight greater than 10,000 to 12,000 (Spectrum Medical Instruments, Los Angeles, Calif.). Each sample was dialyzed against three changes of 400 ml of PBS (pH 7.2) at 4 C. After a 48-h dialysis period, the dialyzed sera were collected aseptically and assayed for hyporeactivity on 2° MEF cells using NDV as the inducer. Also, samples of serum from normal and EMC virus-infected mice that had not been dialyzed but held at 4 C throughout the dialysis period were included in the assay as controls. The results presented in Fig. 3 demonstrate that no loss of biological activity resulted due to dialysis, and, therefore, SHF was assumed to have a molecular weight greater than 10,000.

Centrifugation of SHF at 10,000 × g for 2 h. The previous study indicated that the hyporeactive factor present in serum from EMC virus-infected mice had a molecular weight greater than 10,000. To further estimate its molecular weight and to determine if the factor was incomplete or noninfectious virus, samples of serum from normal uninfected and EMC virus-infected mice were diluted 1:5 in MEM and centrifuged at 40,000 rpm for 2 h at 4 C in a model L Spinco ultracentrifuge using a number 40 rotor. After centrifugation, the top two-thirds of supernatant fluid was removed from each tube and assayed for hyporeactivity on 2° MEF cells using NDV as the inducer. In this assay a

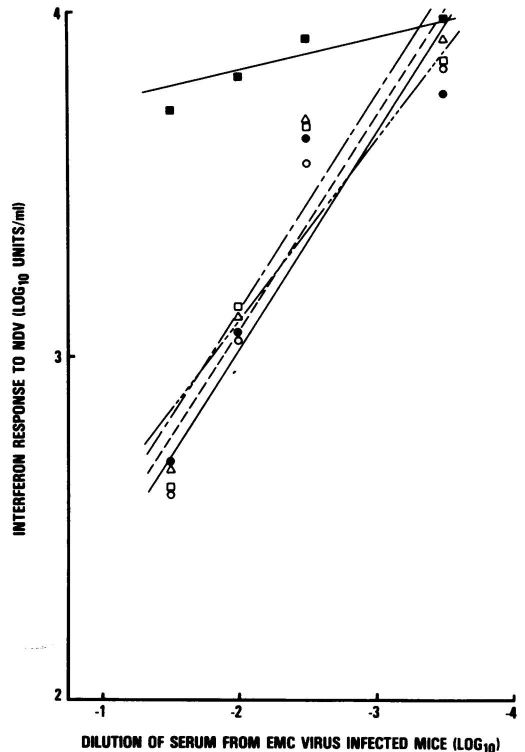

FIG. 2. *Stability of SHF at 37 C. Interferon response induced by NDV in 2° MEF cells treated with serum from normal or EMC virus-infected mice (SHF). Symbols:* O, *Unheated control; SHF held at 37 C for:* □, *1 h;* Δ, *2 h;* ●, *4 h; and* ■, *unheated normal mouse serum.*

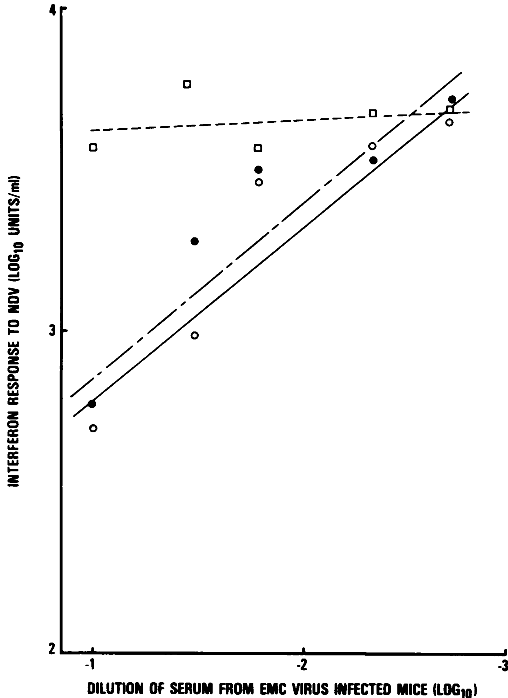

FIG. 3. *Dialysis of SHF for 48 h at 4 C. Interferon response induced by NDV in 2° MEF cells treated with normal mouse serum (NMS) or serum from EMC virus-infected mice (SHF). Symbols: O, SHF, dialyzed 48 h; ●, SHF, 4 C, 48 h; □, NMS, dialyzed 48 h.*

sample of uncentrifuged serum from normal or EMC virus-infected mice served as controls. Ultracentrifugation under these conditions had no detectable effect on the biological activity of the hyporeactive factor (Fig. 4). Although there was some difference between the slope of the lines describing the inhibition created by centrifuged and uncentrifuged samples, the difference was mainly due to the variation in the interferon response of cells treated with the 1:10 dilution of each sample. The interferon response of cells treated with the other dilutions of each sample were almost equal, and the difference between the slope of the two lines was negligible.

pH stability of SHF. As a means of further characterizing SHF, samples of serum from normal and EMC virus-infected mice were diluted 1:5 in MEM and the pH was adjusted to 2, 3, 10, 11, or 7.2, using 2 N HCl or 2 N NaOH (Fig. 5). Samples were held at these pH values for 48 h at 4 C, and then each sample was returned to 7.2 and frozen at −20 C until assayed for hyporeactivity on 2° MEF cells using NDV as the inducing virus. The results presented in Fig. 5 indicate that the hyporeactive factor was stable for at least 48 h at a pH as low as 2 or as high as 11. Using a linear regression analysis of variance to determine the line of best fit, no significant difference was seen between the amount of interferon induced in the cells treated with each dilution of pH-treated or untreated SHF. Normal mouse serum did not significantly impair the ability of the MEF cells to produce interferon and, although not shown, pH treatment of normal mouse serum did not affect the amount of interferon induced in cells treated with these preparations.

Trypsin and RNase sensitivity of SHF. To further determine the chemical nature of SHF, serum from normal or EMC virus-infected mice was diluted 1:5 in Hanks balanced salt solution and 50 μg of trypsin per ml (3× crystallized; Worthington Biochemical Corp., Freehold, N.J.), 50 μg of ribonuclease A (RNase) per ml (Worthington Biochemical), or 0.2 ml of PBS was added to each tube. The mixtures were incubated in a 37 C water bath for 1 h. As controls, a solution of poly(I:C) (100 μg/ml; PL Laboratories, Milwaukee, Wis.) and a sample of

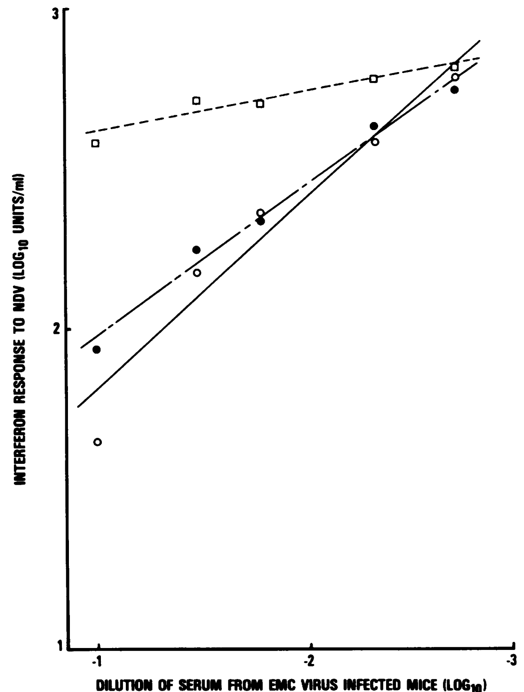

FIG. 4. *Centrifugation of SHF at 100,000 × g for 2 h. Interferon response induced by NDV in 2° MEF cells treated with serum from normal (NMS) or EMC virus-infected mice (SHF). Symbols: O, SHF, 100,000 × g; □, NMS, 100,000 × g; ●, SHF, 4 C, 2 h.*

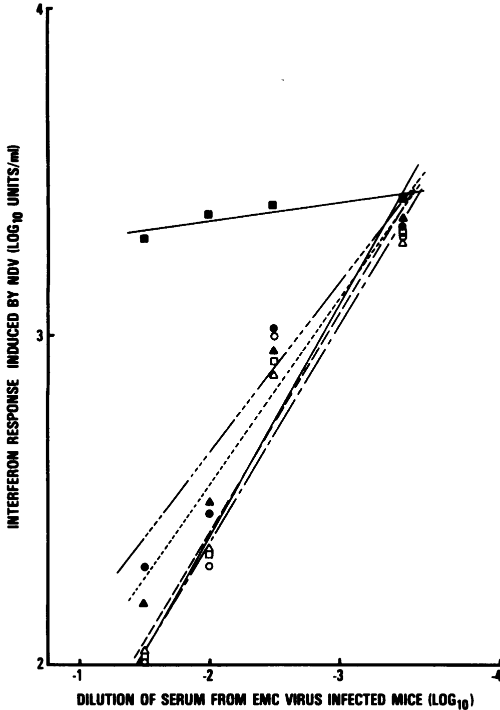

FIG. 5. *Stability of SHF at a pH of 2.0 to 11.0 for 2 days at 4 C. Interferon response induced by NDV in 2° MEF cells treated with serum for normal (NMS) or EMC virus-infected mice (SHF). Symbols: SHF held at: ▲, pH 7.2; ○, pH 3.0; □, pH 2.0; △, pH 10.0; ●, pH 11.0; and ■, NMS, pH 7.2.*

mouse serum interferon (induced by Tilorone hydrochloride and previously characterized as interferon) were also treated with trypsin, RNase, and PBS. After the 1-h incubation period, an equal volume of MEM plus 10% fetal calf serum was added to each tube, and samples were mixed and frozen at −20 C until assayed for biological activity. The serum samples from EMC virus-infected mice were assayed for hyporeactivity on 2° MEF cells using NDV as the interferon inducer. Interferon samples treated with RNase, trypsin, and PBS were assayed on L_{929} using VSV as the indicator virus. Biological activity of poly(I:C) was determined by its ability to create an antiviral state on primary rabbit kidney cells, using VSV as the indicator virus in a plaque reduction assay. This assay system is extremely sensitive to the action of poly(I:C), being able to detect less than 1 μg/ml.

The results indicate that SHF was sensitive to the action of trypsin but resistant to RNase (Fig. 6, 7). Cells treated with a 1:10 dilution of serum from EMC virus-infected mice that had been incubated with PBS produced only 10 U of interferon per ml of growth medium in response to NDV, but cells treated with the same dilution of serum that had been trypsin treated produced 400 U of interferon per ml (Fig. 6). The amount of interferon induced by NDV in 2° MEF cells was, on the other hand, approximately the same in cells treated with each dilution of serum from infected mice that had been incubated with RNase or PBS (Fig. 7). Neither RNase nor trypsin treatment altered the effect of normal mouse serum on the interferon response of cells. Although not shown, trypsinization destroyed greater than 95% of the biological activity of the interferon preparation but had no effect upon the ability of poly(I:C) to create an antiviral state in rabbit kidney cells. RNase treatment, as expected, had the opposite effect: complete destruction of any detectable biological activity of poly(I:C) and no effect on the interferon control. These results indicate that trypsin selectively destroyed the biological activity of protein or polypeptides while not affecting ribonucleic acid integrity, and RNase

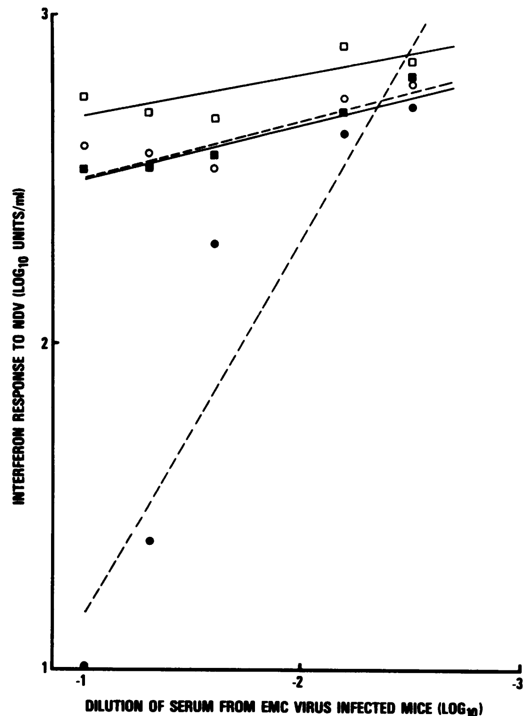

FIG. 6. *Stability of SHF when incubated with 50 μg of trypsin per ml for 1 h at 37 C. Interferon response induced by NDV in 2° MEF cells treated with serum from normal (NMS) or EMC virus-infected mice (SHF). Symbols: ●, SHF + PBS; ○, SHF + trypsin; ■, NMS + PBS; □, NMS + trypsin.*

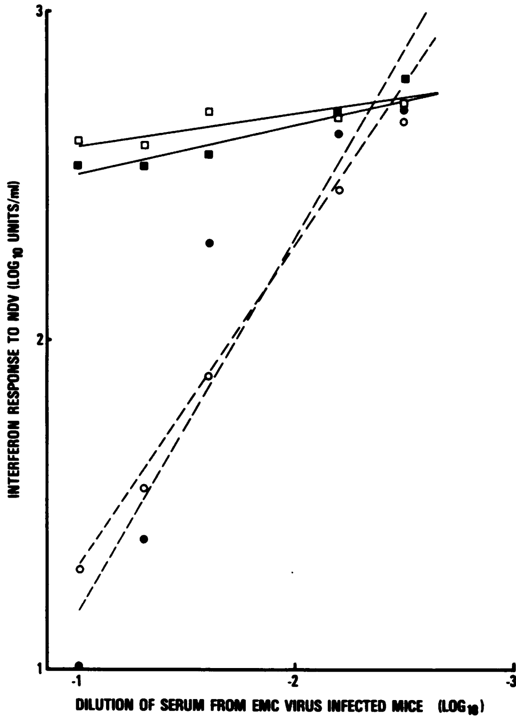

FIG. 7. *Stability of SHF when incubated with 50 μg of RNase per ml for 1 h at 37 C. Interferon response induced by NDV in MEF cells treated with serum from normal (NMS) or EMC virus-infected mice (SHF). Symbols:* ●, *SHF + PBS;* ○, *SHF + RNase;* ■, *NMS + PBS;* □, *NMS + RNase.*

destroyed ribonucleic acid activity without affecting the biological activity of proteins.

G-200 chromatography of serum from EMC virus-infected mice. Using Sephadex chromatography, the molecular weight of the hyporeactive factor in serum from EMC virus-infected mice was determined. Fractionation of serum utilized a 60- by 1.6-cm column (Pharmacia) with a 48- by 1.6-cm bed volume of Sephadex G-200 superfine (Pharmacia), using 0.1 M tris-(hydroxymethyl)aminomethane-hydrochloride buffer at pH 8.0 with 0.2 M NaCl and 0.02% sodium azide. After the addition of 2 ml of serum from EMC virus-infected mice, 0.85-ml fractions were collected and the absorbance of each fraction was read using a Cary model 11 UV spectrophotometer (Applied Physics Corp.) at 280 μM. Fractions were then pooled into 12 equal pools. These samples were dialyzed for 24 h against PBS at 4 C and were then frozen at −20 C until assayed for hyporeactivity on 2° MEF cells using NDV as the inducer. The only pool that demonstrated any hyporeactivity was that comprised of fractions 61–69 (Fig. 8). Cells treated with dilutions of this pool ranging from 1:2 to 1:32 produced less than 50% of the interferon response of cells treated with MEM or comparable dilutions of normal mouse serum (1:2 = 230 U/ml; 1:32 = 950 U/ml; MEM control = 2,200 U/ml; normal mouse serum 1:2 control = 1,500 U/ml). Higher dilutions of this pool had no significant effect on the amount of interferon induced by NDV.

The molecular weight of the hyporeactive factor was determined using a calibration curve, established by chromatography of several proteins of known molecular weight. After the serum, described above, had passed through the column, the column was rinsed with approximately 100 ml of tris(hydroxymethyl)aminomethane-hydrochloride buffer, and then 2 ml of a solution containing purified horse gamma globulin (Upjohn), tryptophan (Schwarz/Mann), bovine serum albumin (Schwarz/Mann), and cytochrome *c* (Schwarz/Mann) (5 mg/ml of each) was chromatographed. The elution volume of each of these proteins was determined, and, using the formula Kav = $V_e - V_o/V_t - V_0$ where V_e is the elution volume, V_t is the total bed volume, and V_0 is the void volume, the Kav of each was plotted against the $\log_{10}$ of their molecular weight (Fig. 9). Using a linear regression analysis of variance, the line of best fit was determined, and the molecular weight of the hyporeactive factor was estimated to be between 20,000 and 40,000 using the Kav of fractions 61 and 69.

To check the validity of these calculations, the molecular weight of the third major peak in Fig. 8 was calculated using its elution coefficient (Kav) and Fig. 9. Since this fraction was

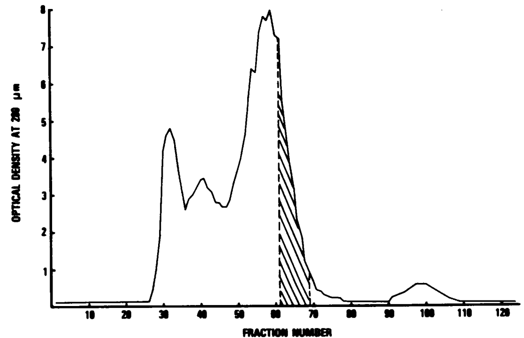

FIG. 8. *Fractionation of serum from EMC virus-infected mice through Sephadex G-200. Fractions were pooled into 12 equal pools and assayed for hyporeactivity on 2° MEF cells using NDV as the inducer. Cross-hatched area represents the only pool that significantly inhibited the cellular interferon response.*

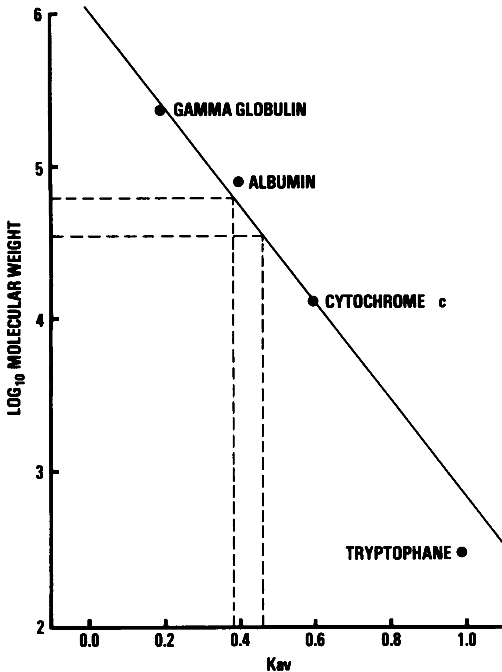

FIG. 9. *Calabration curve of the column described in Fig. 8, estimated by fractionation of a solution containing several proteins of known molecular weight. Using the Kav of each, the molecular weight of the pool containing SHF was estimated and is designated by the two dashed lines.*

serum albumin, it should have a molecular weight of 69,000; using Fig. 9, it had a calculated molecular weight of 67,000.

Serum from EMC virus-infected mice was chromatographed twice, and in each case the only hyporeactivity observed was in the 20,000 to 40,000 molecular weight range.

Effect of anti-interferon antibody on SHF. Although no interferon could be detected in the preparation of serum from EMC virus-infected mice used in these studies, the previous data indicate that SHF has many of the same physicochemical properties as interferon.

To determine if the hyporeactive factor in serum from EMC virus-infected mice was an altered form of interferon that lacked antiviral activity, the effect of anti-interferon antibody on the biological activity of SHF was investigated. Rabbit anti-interferon globulin was obtained through the Antiviral Substances Program of the NIH. Partially purified, NDV-induced, L-cell interferon had been used as the immunogen, and sera had been inactivated at 56 C for 30 min. The globulin fraction had been precipitated and purified by sequential absorption (i.e., calf serum and L-cell extracts) to remove extraneous antibody.

Sequential dilutions of serum from EMC virus-infected mice or murine serum interferon which had been induced by EMC virus were added to equal volumes of each dilution of anti-interferon globulin or MEM (Table 1). Tubes were shaken and held at 37 C for 0.5 h. SHF-antibody mixtures were then assayed for hyporeactivity on 2° MEF cells using NDV as the inducing virus, and each interferon-antibody mixture was assayed for biological activity using a VSV plaque reduction assay on L_{929} cells. The data presented in Table 1 indicate that anti-interferon globulin had no effect on the biological activity of SHF but effectively neutralized the antiviral activity of the EMC virus-induced interferon. For example, cells treated with SHF which had been incubated with an equal volume of the 1:50 dilution of anti-interferon globulin produced 900 U of interferon per ml, compared with 700 U/ml in cells treated with SHF that had been incubated with MEM. In response to NDV, MEM-treated control cells produced from 5,400 to 8,600 U of interferon per ml. The 1:50 dilution of anti-interferon globulin, however, completely neutralized the antiviral activity of 50 U of EMC virus-induced interferon. Although now shown, anti-interferon globulin neutralized the antiviral activity of the international murine interferon standard as efficiently as the EMC virus-induced interferon. These data suggest that SHF and EMC virus-induced interferon are not immunologically related and, therefore, are probably not the same, or altered forms of the same, molecule.

DISCUSSION

Serum from EMC virus-infected mice taken 96 h after injection of virus contains a factor capable of transferring hyporeactivity to otherwise normal murine cells in vitro (12). The results of studies in this report indicate that the factor is protein in nature, since it was sensitive to proteolytic action of trypsin but resistant to RNase. Heating at 56 C for as little as 8 min destroyed greater than 90% of the biological activity of SHF. At 37 C, however, SHF was stable, with no detectable loss in hyporeactivity during a 4-h period. The hyporeactive protein in serum from infected mice was stable over a pH range of 2 to 11 for 48 h at 4 C and had, by fractionation on Sephadex G-200, an estimated molecular weight of from 20,000 to 40,000. These conclusions are supported by the observation that SHF was non-dialyzable and was not sedimented when centrifuged at 100,000 $\times g$ for 2 h. Serum from EMC virus-infected mice has previously been reported to be species

TABLE 1. *Neutralization of interferon, but not SHF, anti-interferon globulin*

Dilution of SHF	MEM	Dilution of anti-interferon globulin[a]					
		1:50	1:100	1:500	1:1,000	1:5,000	1:1,000
	Interferon levels induced by NDV (U/ml)						
1:10	700[b]	900	1,100	850	900	1,000	950
1:50	1,700	1,600	1,200	1,800	1,800	2,000	1,100
1:100	3,800	3,500	4,300	4,500	4,200	4,500	4,800
1:500	4,600	4,300	4,400	4,800	5,000	5,000	6,000
MEM	8,600	7,000	7,600	8,100	7,100	6,500	5,400
Interferon (U/ml)	Avg no. of VSV plaques/plate						
50	0[c]	40	30	26	22	0	0
10	8	40	42	40	30	24	8
5	14	32	44	32	28	36	18
1	28	38	40	40	38	30	32
MEM	34	42	36	38	42	38	40

[a] Equal volumes of each dilution of serum from EMC-infected mice (SHF), interferon (induced by EMC (virus), or MEM were added to each dilution of anti-interferon globulin or MEM and incubated at 37 C for 30 min. These mixtures were then assayed for hyporeactivity on 2° MEF cells using NDV as the inducer, or for interferon using a VSV plaque reduction assay.

[b] Interferon levels (units/milliliter) induced by MEF cells treated with SHF-ab mixtures.

[c] Average number of VSV plaques per L929 plate (three plates per dilution) treated with interferon-ab mixtures.

specific in the sense that it rendered three types of murine cells hyporeactive to interferon induction by NDV, but had essentially no effect on the interferon responsiveness of heterologous cells, including rabbit, chicken, and human cells (12). Also, SHF had no effect on the sensitivity of murine cells to the antiviral action of interferon (12). Although these results suggest similarities between SHF and interferon, no neutralization of the biological activity of SHF was observed when incubated with anti-interferon globulin that could neutralize the antiviral activity of EMC virus-induced interferon.

When cells are challenged with interferon inducers they often become refractory to new stimulation. Also, cells exposed to interferon prior to induction can exhibit either an enhanced (primed) or reduced (hyporeactive) interferon response (2, 4, 6, 7, 9, 13, 15). Although a number of in vitro systems have been used in an attempt to elucidate the mechanism of the refractory state, no definitive explanation has yet been achieved. Different hypotheses have been proposed to explain the mechanism of the refractory state; an inhibitor acting as a suppressor of interferon production might appear with interferon or the antiviral state in vitro and could feed back on interferon synthesis through an interferon-induced protein, or interferon itself might modulate its own production. Borden and Murphy (1), Borden et al. (E. C. Borden, E. U. Prochownik, and W. A. Carter, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1972, V22, p. 189), and Byrd et al. (D. M. Byrd, K. Chadha, and W. A. Carter, Progr. Abstr. Intersci. Conf. Antimicrob. A. Chemother., 13th, Washington, D.C., Abstr. 196, 1973) have suggested that an inhibitor with the same physicochemical properties as interferon was produced coincidentally with interferon, and that interferon preparations harvested early after inducer contained little or no hyporeactive factor whereas interferon harvested later contained high levels of hyporeactive factor. Furthermore, there was no apparent relationship between the amount of interferon or hyporeactive factor in any of their preparations. Using various purification techniques, however, they were unsuccessful in separating the postulated hyporeactive factor from interferon. Rousset (10) likewise found that interferon or an interferon-like material was apparently responsible for hyporeactivity. Vilcek et al. (14), however, have reported that the addition of actinomycin D a short time after induction potentiated the interferon response of rabbit kidney cells, suggesting that another protein whose synthesis is induced by high levels of interferon was responsible for cellular control of interferon production (hyporeactivity).

Although our experiments used an entirely different system than any previously reported, and production of the hyporeactive factors was an in vivo process using an active virus infection of mice, the possibility exists that the SHF characterized in this paper and that reported previously (Borden et al., Abstr. Annu. Meet. Amer. Soc. Microbiol. 1972, V22, p. 189; Byrd et al., Progr. Abstr. Intersci. Conf. Antimicrob. Ag. Chemother., 13th, Washington, D.C., Abstr. 196, 1973) in other systems may be the same. Using various physical and chemical criteria, the only difference between SHF and murine interferon was that hyporeactive factor was not neutralized by anti-interferon globulin whereas EMC virus-induced interferon was, suggesting that they are similar but unrelated molecules. It should be further emphasized that no interferon activity, as measured by using a plaque reduction assay system, could be detected in the serum from EMC virus-infected mice used in these studies. Cells treated with a 1:20 dilution of serum from EMC virus-infected mice developed no antiviral activity, yet produced less than 5% of the interferon levels induced by NDV in MEM- or normal mouse serum-treated cells. Using an in vivo system, an interferon-free preparation of SHF was obtained by waiting until the animal had cleared the interferonemia before serum was drawn. Other systems which have employed in vitro production of hyporeactive factor have in every case had interferon in the preparation, and differentiation of the action of hyporeactive factor and interferon has not been possible due to the apparent close similarity of the two molecules (Borden et al., Abstr. Annu. Meet. Amer. Soc. Microbiol. 1972, V22, P 189; Byrd et al., Progr. Abstr. Intersci. Conf. Antimicrob. Ag. Chemother., 13th, Washington, D.C., Abstr. 196, 1973).

Although the serum hyporeactive factor described in this report may not be the same hyporeactive factor whose existence has previously been postulated, the possibility is intriguing. Whether the factor present in the serum of EMC virus-infected mice is a virus-specific substance designed to inhibit the interferon component of host defense or is a specific (natural) cellular or physiological mechanism responsible for control of interferon production is at the present time unknown. Studies are under way to purify, further characterize, and determine the mechanism of action of SHF. Such information would seem essential to understanding how or why animals develop hyporeactivity to interferon induction and could in turn be beneficial in developing methods of overcoming a potentially severe limitation to the use of interferon inducers as effective therapeutic antiviral agents, as well as further defining conditions that may influence the virulence of specific virus infections.

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