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Hyporeactivity Due to Infection: Recognition of a Transferable Hyporeactive Factor in the Serum of Encephalomyocarditis Virus-Infected Mice

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As previously reported, mice infected with encephalomyocarditis (EMC) virus progressively develop a state of hyporeactivity to interferon induction as evidenced by an 80% reduction in serum interferon levels produced in response to four different inducers administered 96 h after initiation of infection. In the current report, this hyporesponsiveness was further documented in vitro in peritoneal exudate cells harvested at the time of maximal hyporeactivity (96 h) during EMC virus infection. These peritoneal cell preparations (containing no detectable interferon or virus) produced significantly less interferon than cells from uninfected mice following exposure to Newcastle disease virus or Chikungunya virus. More importantly, hyporeactivity could be transferred to normal peritoneal cells incubated in serum (containing no detectable interferon or virus) from EMC virus-infected mice during the hyporeactive period. Incubation of peritoneal exudate cells with up to a 1:200 dilution of serum from hyporeactive animals still resulted in a significant reduction in the interferon response induced by Newcastle disease virus or Chikungunya virus. Furthermore, it could be shown that this serum hyporeactive factor was effective in mouse cells but not in rabbit, chicken, or human cells.

Mice infected with encephalomyocarditis (EMC) virus progressively develop a severe state of hyporeactivity to interferon induction (7). By 96 h after infection with EMC virus, circulating interferon levels induced by poly (I:C), Newcastle disease virus (NDV), or Tilorone hydrochloride were reduced by 80% or more. Other experimental virus infections have also been reported to create a similar state of hyporesponsiveness to interferon induction including Friend leukemia virus (2), lymphocytic choriomeningitis virus (4), and cytomegalovirus (5). If such a condition develops in man as a result of virus infection, it could represent a major limitation to the use of interferon inducers as effective therapeutic agents.

The purpose of the studies presented in this report was to investigate mechanisms responsible for the state of hyporeactivity to interferon induction created by infection. Initially, the capacity of peritoneal macrophages and lymphocytes from hyporeactive animals to produce interferon in vitro was defined. The occurrence of hyporeactivity in cells which were not infected with the virus suggested the possibility that a circulating mediator of hyporeactivity might be present in virus-infected, hyporeactive animals. This hypothesis was supported by the demonstration that the interferon response of normal cells could be suppressed by incubation in serum from EMC virus-infected mice.

MATERIALS AND METHODS

Mice. Female CD-1 mice were obtained from Charles Rivers Breeding Laboratories (Brookline, Mass.) and 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 for at least 1 week to allow them to adjust to the new environment before being used in experiments.

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.

Primary rabbit kidney (RK) cells were prepared from 350-g suckling rabbits. Animals were sacrificed and kidneys were removed, decapsulated, and trypsinized (0.25%, 1:250 Difco). Cells were distributed in 2-ml quantities in Eagle minimal essential medium (MEM) (4×10^5/ml) to 35-mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.). Confluent monolayers were obtained in 5 days.

Primary embryonic chicken kidney (CK) cells were

prepared from 19-day-old embryonated hen eggs. Embryos were removed aseptically, and kidneys were removed and trypsinized. Cells were suspended in MEM and distributed at 8×10^5 cells/ml, 2 ml per 35-mm petri dish. Confluent monolayers were obtained in 2 days.

Human foreskin fibroblasts (HFF) were obtained from R.D. Hamilton (The Upjohn Co., Kalamazoo, Mich.). The cells had been carried for approximately 15 passages at the time of use. Cells were maintained in 75-cm^2 T flasks (Falcon Plastics), trypsinzed, suspended in MEM at a concentration of 2×10^4 cells per ml, and distributed (2 ml/plate) into 35-mm plastic petri dishes. Confluent monolayers were obtained in 5 days.

Secondary mouse embryo fibroblasts (MEF) were obtained by sacrificing pregnant CD-1 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 MEM at 5×10^4 cells/ml and distributed into 32-ounce 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. MEM (Auto-Pow, Flow Laboratories, Rockville, Md.) contained 5% fetal calf serum (GIBCO, Grand Island, N.Y.) and 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 NDV was originally obtained from S. Baron (National Institutes of Health). The stock NDV preparation used in these experiments was propagated in embryonated hens eggs which had been injected by the allantoic route and had a titer of 3.7×10^9 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.). The stock preparation of VSV used in these studies was propagated in primary chicken embryo cell monolayers and had a titer of 2.5×10^8 PFU/ml when assayed in L_{929} mouse cells.

The strain of EMC virus was a large-plaque variant originally obtained from K. K. Takemoto (National Institutes of Health). Suckling mice were injected by the intracerebral route; brains were removed when the animals were moribund and homogenized. A 10% suspension of this material containing 5×10^6 PFU/ml was used as a stock pool.

Chickungunya virus (CV) was obtained from P. Russell, Walter Reed Medical Center, and was propagated in primary chicken embryo cells. The stock preparation used in these studies titered 5×10^8 PFU/ml on chicken embryo cells.

Interferon assay. Confluent monolayers of L_{929}, HFF, RK, or CK cells grown in 35-mm plastic petri dishes (Falcon Plastics) were treated with 1 ml of the appropriate interferon dilution overnight at 37 C. The plaque reduction assay used VSV as the challenge virus and has been previously described (7). An internal laboratory murine interferon standard was included with each mouse interferon assay, and the sensitivity of our assay was compared with the International Murine Standard obtained from the National Institutes of Health. In our system, the International Murine Standard had a titer of approximately 400 U/ml compared with its accepted titer of 500 U/ml.

RESULTS

In Vitro interferon response of cells from EMC virus-infected mice. To investigate the ability of cells from EMC virus-infected hyporeactive mice to respond to interferon induction in vitro, a group of 20 mice was injected intraperitoneally with 1,000 PFU of EMC virus. Mice were sacrificed 100 h after infection and peritoneal exudate cells (PEC) were harvested, centrifuged, and resuspended at a concentration of 10^6 cells/ml in fresh complete growth medium (MEM). No virus could be detected in any of the PEC preparations using either a lysate obtained by freeze-thawing the cell preparation or by an infectious center assay on L_{929} cells. Similarly, no evidence of interferon could be found in the medium harvested from the cultures of PEC from EMC virus-infected mice. Thus, there was no evidence of infectious virus or interferon in the macrophage-lymphocyte population utilized in these experiments. The PEC preparation was incubated overnight at 37 C in 35-mm plastic petri dishes (2 ml/plate). Cells from EMC virus-infected mice were challenged with either 4×10^7 PFU of NDV, 10^7 PFU of CV, or 0.1 ml of MEM. The cultures were then returned to the incubator for 18 to 24 h when growth medium was harvested and assayed for interferon. The results from one representative experiment are summarized in Table 1. Peritoneal cells from infected mice produced only 150 U of interferon in response to NDV and less than 100 U in response to CV. This represented less than 4 and 10%, respectively, of the interferon levels induced in control cell populations from uninfected mice. The observed reduction could not be attributed to a difference in the type or distribution of cells in each preparation, since each contained approximately equivalent percentages of lymphocytes and macrophages. These data demonstrate that macrophages and lymphocytes from EMC virus-infected mice manifest a hyporesponsive state when stimulated to produce interferon in vitro.

Transfer of hyporeactivity by serum for virus-infected mice. The observation that

TABLE 1. *Interferon levels induced in vitro in PECs from EMC virus-infected and uninfected mice*

Source of PECs	Inducer	Interferon levels (U/ml)
EMC-infected mice[a]	NDV	150
EMC-infected mice	CV	<100
EMC-infected mice	MEM	<50
Normal mice	NDV	3,900
Normal mice	CV	1,000
Normal mice	MEM	<50

[a] PEC preparations from EMC virus-infected mice contained no detectable virus or interferon before inducer challenge.

PECs from EMC virus-infected mice reflected the hyporesponsiveness of the intact animal was particularly interesting since neither EMC virus nor interferon could be detected in the cell preparations. These data suggest that some other factor might be responsible for mediating the observed hyporeactivity. To investigate this possibility, mice were injected by the intraperitoneal route with approximately 1,000 PFU of EMC virus and were bled by cardiac puncture 100 h later at the time of maximal hyporeactivity in vivo. Serum was subsequently collected and found to contain no detectable interferon or EMC virus when assayed on L_{929} cells. Serum, 0.1 ml, from EMC virus-infected mice was then added to 35-mm petri dishes containing peritoneal exudate cells from uninfected mice which were maintained in 2 ml of complete growth medium. Sets of control cultures received 0.1 ml of either serum from uninfected control mice or MEM. All cell cultures were returned to 37 C for 18 h and then either 4×10^7 PFU of NDV, 10^7 PFU of CV, or 0.1 ml of MEM was added to each plate. Growth media from subgroups of three plates from each experimental group were harvested 24 h after addition of inducer and were frozen at −20 C until assayed for interferon.

As little as 0.1 ml of serum (1:20 dilution) from EMC virus-infected mice depressed the NDV-induced interferon response from 3,000 U/ml in cultures treated with normal mouse serum to 100 U/ml in those treated with serum from hyporeactive animals (Table 2). The interferon response to CV was similarly reduced from 1,000 U/ml in controls to less than 100 U/ml. These data suggest that serum from EMC virus-infected mice contains a factor or factors capable of transferring hyporeactivity to interferon induction to otherwise normal cells.

These original observations were then extended to determine if serum from EMC virus-infected mice could reduce the ability of other murine cell lines as well as cells from other animal species to respond to interferon induction. Groups of three plates containing confluent monolayers of L_{929}, secondary MEF, CK, RK, HFF, or mouse PEC received 1 ml of a serial dilution of serum from EMC virus-infected mice, normal mouse serum, or MEM. Following an identical protocol as in the previous series of experiments, interferon production was induced with either NDV or CV. Culture fluids from murine, rabbit, chicken, and human cells were assayed for interferon on cells from the appropriate species. Serum from EMC virus-infected mice inhibited the interferon response of each of the three types of murine cell cultures but had essentially no effect on the response of rabbit, chicken, or human cells (Fig. 1). In this figure, each line was plotted using a linear regression analysis of variance and each line had a correlation to the data points of at least 98%. Of the murine cells tested, secondary MEF and PECs appeared to be the most sensitive to the action of the hyporeactive factor. When diluted as high as 1:200, the interferon response induced by NDV was reduced by greater than 50% when compared with the control groups (PEC, 4,000 U/ml versus 1,800 U/ml; MEF, 4,500 U/ml versus 1,100 U/ml). L_{929} cells did not appear to be sensitive as the other murine cells, and although the difference was not statistically significant, a 50% inhibition of control interferon levels was observed in cells treated with a 1:100 dilution of serum from EMC-infected mice (3,200 U/ml versus 9,000 U/ml). None of the heterologous cell systems used in these studies demonstrated

TABLE 2. *Effect of serum from EMC virus-infected mice on the ability of PECs from uninfected mice to produce interferon*[a]

Treatment	Inducer	Interferon levels (U/ml)
Serum from EMC[b]-infected mice	CV	<50
	NDV	100
	MEM	<50
Normal mouse serum	CV	1,000
	NDV	3,000
	MEM	<50
MEM	CV	1,500
	NDV	2,000
	MEM	<50

[a] 0.1 ml of either serum from EMC virus-infected mice, normal mouse serum, or MEM was added to each plate.

[b] Serum was collected from mice 100 h after infection with EMC virus and contained no detectable interferon or virus.

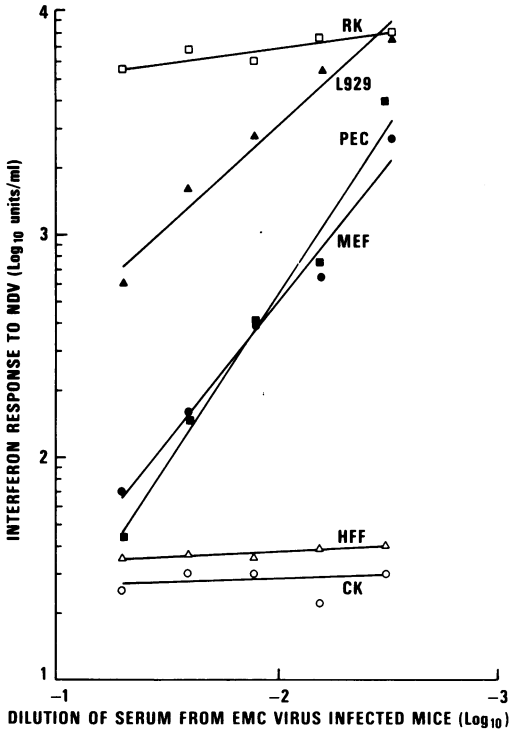

FIG. 1. *Suppression of the interferon response of murine cells (L_{929}, MEF, PEC), but not rabbit (RK), human (HFF), or chicken (CK) cells by the hyporeactive factor in serum from EMC virus-infected mice.*

a significantly reduced interferon response after exposure to serum from infected mice. In each case, a slight reduction in the amount of interferon induced by NDV in cells treated with the lowest serum dilutions was observed. This slight reduction was also observed in cells, both murine as well as each of the other species, treated with comparable dilutions of serum from normal uninfected mice. These results confirm the occurrence of a circulating factor which can mediate hyporeactivity and further demonstrate that its presence may be detected at moderately high dilutions of serum from EMC virus-infected animals.

Effect of serum from EMC virus-infected mice on sensitivity of MEF cells to the action of interferon. One explanation for the reduced interferon response of murine cells exposed to serum from EMC virus-infected mice might be toxicity, although the fact that a similar toxicity was not observed in cells from other animal species does not support such a theory. To further investigate this hypothesis and to determine the effect of serum from hyporeactive EMC virus-infected mice on other parameters of cellular integrity, the effect of serum from infected mice on the sensitivity of secondary MEF cells to the antiviral action of interferon was determined. Serial dilutions of serum from normal or EMC virus-infected mice were made in MEM. Growth medium was removed from confluent secondary MEF cell cultures grown in 35-mm petri dishes, and 1 ml of one of several dilutions of serum from normal mice or EMC-infected mice was added to sets of three plates. After overnight incubation at 37 C, the monolayers were washed with 2 ml of PBS and each plate received 1 ml of an appropriate dilution of murine serum interferon which had previously been induced by Tilorone hydrochloride and characterized as murine interferon. A standard plaque reduction interferon assay was then performed using VSV as the indicator virus. Control sets of plates treated with each dilution of serum from infected and normal mice were similarly incubated overnight at 37 C. The monolayers were then washed with 2 ml of PBS and 1 ml of MEM was added to each plate, followed by 4×10^7 PFU of NDV or 0.1 ml of MEM. Cell cultures were returned to the incubator and culture fluids were harvested 24 h later and frozen at −20 C until assayed for interferon. The results from this series of experiments are illustrated in Table 3. No inhibitory effect on the sensitivity of secondary MEF cells to the action of interferon could be detected. Although the differences are not significant, cells exposed to the lowest dilution (1:20) of

TABLE 3. *Effect of serum from EMC virus-infected mice on the senstivity of secondary MEF cells to the action of interferon*

Dilution of serum from EMC virus-infected mice (1 ml/plate)	Titer of a standard mouse interferon preparation (U/ml)[a]	Interferon response to NDV (U/ml)[b]
1:20	25,000	100
1:200	20,000	1,300
1:2000	12,000	5,100
1:20,000	16,000	4,300
NMS[c]	12,000	4,100
MEM	11,700	4,600

[a] After overnight incubation with serum from EMC virus-infected or normal mice, monolayers were washed with PBS and received appropriate dilutions of interferon. A standard plaque reduction assay was then performed.

[b] After overnight incubation with serum from EMC virus-infected mice, monolayers were washed with PBS and 4×10 PFU of NDV was added to each plate. Growth medium was harvested 24 h later and assayed for interferon.

[c] Normal mouse serum (1:20 dilution).

serum from EMC virus-infected mice appeared to be the most sensitive to the development of antiviral resistance due to interferon. In these cells, the interferon preparation titered greater than 25,000 U/ml compared with a titer of 12,000 U/ml in control cells exposed to normal mouse serum or MEM. A matched set of cell cultures which had been treated in an identical fashion with the same serum dilutions was shown to have the predicted impairment in their capacity to produce interferon in response to NDV. These results suggest that the inhibitory effect of serum from hyporeactive EMC virus-infected mice was not due to toxicity since cells exposed to the serum showed no morphological alterations, were sensitive to the antiviral action of interferon, and could support the replication of VSV.

DISCUSSION

The development of tolerance or hyporeactivity to repeated injections of interferon inducers has been reported on numerous occasions (1, 3, 6, 8–10). Hyporesponsiveness resulting as a consequence of infection, however, has only recently been extensively investigated. Stringfellow and Glasgow (7) reported that mice infected with EMC virus progressively develop a state of hyporeactivity. Similarly, other reports have indicated that mice infected with Friend leukemia virus (2), cytomegalovirus (5), and lymphocytic choriomeningitis virus (4) have a compromised capacity to produce interferon. Other virus infections may also create a similar situation in experimental animals, and if such a condition develops in man as a consequence of virus infection, it may represent a major limitation to the eventual use of interferon inducers as clinically useful therapeutic agents. Studies designed to investigate mechanisms or explanations for the development of the hyporeactive state that results as a consequence of virus infection would, therefore, seem essential. These observations indicate that at least certain viruses have the capability in inhibiting the host's interferon response and raise the additional question of the potential contribution of this capability to the virulence of the virus. If interferon is one component of host resistance, the ability to suppress this determinate of the host's defenses might be expected to enhance the pathogenicity of the parasite.

The results presented further indicate that peritoneal cells obtained from EMC virus-infected mice at the time of maximal hyporeactivity in vivo reflect that hyporeactivity when challenged in vitro with either of two viral interferon inducers. These results are particularly interesting since neither interferon nor EMC virus could be detected in any of the cell preparations before inducer challenge. Furthermore, the population of cells in each preparation did not significantly differ in terms of the relative percentage of lymphocytes or macrophages or total cell numbers and therefore could not be explained on this basis. The decreased interferon response of cells from EMC virus-infected animals in the absence of detectable virus or interferon suggest that a soluble factor or a product of virus infection might be responsible for mediating the observed hyporeactivity.

Such a factor or factors were demonstrated in the serum of EMC virus-infected mice collected at the time of maximal hyporeactivity in vivo. These serum preparations contained no detectable EMC virus nor interferon, yet treatment of peritoneal cells from normal uninfected mice with dilutions of the serum resulted in a gross impairment of the ability of cells to produce interferon in response to NDV or CV. The suppression of the interferon response of other murine cell lines, L_{929} and MEF, was also observed; however, the hyporeactive factor from infected mice had no effect on the NDV-induced interferon response of human, rabbit, or chicken cells. These results indicate that the hyporeactive activity of serum from EMC virus-infected mice was, at least to this extent, species specific and suggest that impairment of the interferon response was not due to cellular toxicity.

As further evidence that the hyporeactivity transmitted to murine cells was not a result of toxicity, the effect of serum from EMC virus-infected mice on the ability of MEF cells to respond to the action of interferon, by developing antiviral resistance, was investigated by using a plaque reduction assay. Cells treated with serum from EMC virus-infected mice were at least as sensitive to the antiviral action of interferon as were cells treated with MEM or normal mouse serum. This capacity of MEF cells to develop antiviral resistance on exposure to interferon and to support the replication of VSV suggest that the ability of serum from EMC virus-infected mice to transfer hyporeactivity to otherwise normal cells was not a result of cell toxicity.

These results indicate that the state of hyporeactivity observed in EMC virus-infected mice is reflected in vitro by cells taken from these animals and that the hyporesponsiveness that develops due to infection cannot be explained on the basis of viral destruction of specific cell populations necessary for interferon production.

Furthermore, serum from EMC virus-infected mice contains a factor or factors that can inhibit the CV or NDV-induced interferon response of murine cells. Whether the factor present in serum from EMC virus-infected mice is a virus-specific substance with the capacity to inhibit the interferon component of host defense or is a specific cellular or physiological mechanism responsible for the control of interferon production is at the present time unclear. Currently, studies are under way to determine the biological and physicochemical nature and mechanism of action of this factor(s). Further information would seem essential to understanding how or why animals develop hyporeactivity to interferon induction as a consequence of infection and could in turn be beneficial in developing methods of overcoming a potentially severe limitation to the use of interferon inducers as therapeutic antiviral agents.

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