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Treatment of Mice with Polyinosinic-Polycytidilic Polyribonucleotide Reduces T-Cell Involvement in a Localized Inflammatory Response to Vaccinia Virus Challenge

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Mice inoculated intracerebrally with 10^3 PFU of vaccinia virus developed a nonfatal meningitis which was maximal 7 days after challenge. Intravenous administration of an interferon (IFN) inducer, polyinosinic-polycytidilic polyribonucleotide [poly(I)-poly(C)], on days 4 and 6 postinjection was associated with a three- to fourfold decrease in the number of T lymphocytes present in cerebrospinal fluid, reflected primarily by a decreased number of vaccinia virus-specific cytotoxic T-lymphocyte precursors. The lack of a concomitant reduction in the overall cytotoxic activity of cerebrospinal fluid cells directed against virus-infected target cells seemed to be largely due to an increase in natural killer cell activity. IFN was implicated as mediating the effect of poly(I)-poly(C) because high systemic levels of IFN were evident after injection, and neither the magnitude of the inflammatory response nor the T-cell levels were affected when poly(I)-poly(C)-treated mice were also given anti-IFN antiserum. However, the poly(I)-poly(C)-induced IFN did not seem to reduce the localized inflammatory response by affecting viral replication in brain tissue because the vaccinia virus titers present on days 6 through 8 of infection were similar to the titers in phosphate-buffered saline controls. These findings are consistent with either an effect of IFN on T-cell recruitment to the central nervous system or an inhibition of proliferation of cells participating in the response. These findings suggest that there is a potential source of complications for clinical protocols that use IFN or inducers to enhance T-cell function in various disease situations, and this effect of IFN may be a contributing factor to the immunosuppression often associated with many viral infections.

Interferon (IFN) has been shown to modulate immune response to antigenic challenge. In this regard, IFN can significantly retard such T cell-dependent responses as the rejection of allogeneic skin grafts (7), delayed-type hypersensitivity (6, 8), graft-versus-host disease (3, 23), and T-cell proliferation in vitro (22, 34, 35). Recently, it has been demonstrated in mice that high systemic levels of IFN, resulting from either direct administration or induction by the polyinosinic-polycytidilic polyribonucleotide [poly(I)-poly(C)], can cause acute lymphocytopenia in both peripheral blood and thoracic duct lymph (20, 30). This inhibition of lymphocyte circulation can last for up to 3 days and is completely blocked by treatment of the mice with anti-IFN antiserum. Viruses have also been shown to be capable of mediating this effect, which also correlates with the induction of high IFN levels (30, 40). Although IFN has clearly been shown to be an effective antiviral agent at the site of infection (5, 17, 26, 32, 41), excessive levels of IFN may have deleterious effects on the host immune response to the virus. Thus, IFN-mediated inhibition of lymphocyte circulation may be a major contributing factor to the immunosuppression often associated with many viral infections (4, 9, 24, 36). It is therefore important to examine closely the effect of IFN on the development of a localized inflammatory response to viral challenge.

We have recently described a murine model in which intracerebral (i.c.) inoculation with a sublethal dose of vaccinia virus results in progressive meningitis (11, 12). The immune response to the viral challenge can be accurately monitored by analysis of cerebrospinal fluid (CSF) from the cisterna magna, the cells of which can be serologically defined and tested for functional activity. Since the CSF normally contains very few cells, it is an excellent way to observe the development of an inflammatory process. The early stages of the response are clearly dominated by nonspecific natural killer (NK)-like effector cells, whereas cytotoxic T lymphocytes (CTL) become predominant by day 6 postinjection (11). As presented in this report, the induction of high systemic levels of IFN by poly(I)-poly(C) injection significantly reduced the number of cells involved in the inflammatory process and particularly affected the T-cell compartment.

MATERIALS AND METHODS

Mice. C57BL/6J (H-2^b) and (C3H × DBA/2)F_1 (H-2^{kxd}) mice [designated B6 and (C3D2)F_1 mice throughout, respectively] were purchased from Jackson Laboratories, Bar Harbor, Maine.

Medium. RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) plus 10% fetal bovine serum (FBS; Flow Laboratories, Inc., McLean, Va.) was used for all procedures.

Virus. Mice were injected i.c. with 10^3 PFU (30 μl) of the WR strain of vaccinia virus grown in BSC-40 cells (1).

Target cells and cytotoxicity assay. The L (H-2^k) and MC57-G (H-2^b) target cells were first labeled with 0.5 mCi of $Na^{51}CrO_4$ (New England Nuclear Corp., Boston, Mass.) in 50 μl of saline for 1 h at 37°C. The cells were then washed, and a portion was infected with vaccinia virus (1 h at 37°C, 10 PFU per cell) and used for a 7- to 8-h ^{51}Cr release cytotoxicity assay as described previously (1, 11). The

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TABLE 1. Effect of poly(I)-poly(C) injection on the composition of inflammatory cells in the CSF of mice injected i.c. with vaccinia virus[a]

Expt	Mouse strain	Group	Injection	No. of cells ($\times 10^5$) per mouse (% of total cells)			
				Total	Thy-1$^+$	Lyt-1$^+$	Lyt-2$^+$
1	B6 (H-2^b)	A	PBS	3.2	2.6 (81)	NT[b]	1.5 (47)
		B	Poly(I)-poly(C)	1.0	0.4 (40)	NT	0.4 (40)
2	(C3D2)F_1 (H-2^{kxd})	C	PBS	5.6	4.3 (77)	3.1 (57)	3.1 (55)
		D	Poly(I)-poly(C)	2.9	1.0 (35)	0.6 (21)	0.7 (24)
3	(C3D2)F_1	E	PBS	5.1	3.7 (73)	3.5 (69)	2.7 (53)
		F	Poly(I)-poly)(C) and NSS	1.6	1.0 (63)	0.6 (38)	0.4 (25)
		G	Poly(I)-poly(C) and IFN-α	10.0	7.2 (72)	5.6 (56)	4.8 (48)
4	(C3D2)F_1	H	PBS	4.0	3.2 (80)	2.5 (63)	2.1 (53)
		I	PBS and NSS	5.9	4.4 (75)	3.1 (53)	2.5 (42)
		J	PBS and IFN-α	5.0	3.3 (66)	2.2 (44)	1.8 (36)

[a] Mice injected i.c. with 30 μl of PBS as control yielded approximately 100 cells per μl of CSF. The total count is an average for pooled CSF samples from groups of 6 to 37 mice. The Lyt-1 surface marker is also found on a large number of Lyt-2$^+$ cells, so that each antibody detects overlapping populations.

[b] NT, Not tested.

YAC-1 tumor target cells (27) were obtained from G. Trinchieri (Wistar Institute) and were used in a 4-h assay. Results are expressed as [(% experimental release − % SR)/(% MR − % SR)] × 100, where maximum release (MR) and spontaneous release (SR) are determined after incubation in cetrimide-saline solution or medium alone, respectively.

Reagents. The synthetic poly(I)-poly(C) was purchased from P-L Biochemicals, Inc., Milwaukee, Wis., and diluted in phosphate-buffered saline (PBS). The source and use of the sheep anti-mouse IFN (alpha and beta) serum, which was kindly supplied by D. M. Murasko (Medical College of Pennsylvania, Philadelphia), has been described elsewhere (30). Mice were injected intraperitoneally (i.p.) with 1.5×10^6 neutralization units of IFN-α (0.15 ml) or an equal volume of normal sheep serum (NSS) (purchased from a service of the University of Pennsylvania Veterinary Hospital, Philadelphia). Antibody reagents included the monoclonal anti-Thy-1.2 ascites fluid produced by the J1j hybridoma (2), a nonpolymorphic monoclonal rat anti-mouse Lyt-2 (39) (supplied by J. Sprent, University of Pennsylvania, Philadelphia) and anti-Lyt-1.1 antiserum (Accurate Chemical, Westbury, N.Y.). Guinea pig complement (1:25 dilution; Flow Laboratories, Inc., McLean, Va.) and rabbit complement (1:80 dilution; provided by J. Sprent) were combined for antibody treatments and cell phenotyping.

Inflammatory cells. Mice were anesthetized with an i.p. injection of 2,2,2-tribromoethanol (Avertin; Aldrich Chemical Co., Milwaukee, Wis.) and exsanguinated from the axilla. CSF samples were then aspirated from the cisterna magna (10, 11) and pooled for each group in medium. As required, cells were treated before assay with monoclonal rat anti-mouse Lyt-2 plus complement. In addition, cells were phenotyped in the presence of complement with either anti-Thy-1.2, anti-Lyt-2, or anti-Lyt-1.1 antibodies in a standard trypan blue exclusion assay (29). Results were expressed as cytotoxic indices and calculated as [(% dead with antibody − % dead without antibody)/(100% − % dead without antibody)] × 100.

Limiting-dilution cultures. The methods for the limiting-dilution analysis have been described in detail previously (12, 25, 38). Briefly, CSF cells were serially diluted into conical-bottom 96-well microtiter plates (Linbro) and stimulated with irradiated (1,500 rads) C3H (H-2^k) spleen cells that had been exposed to UV-irradiated (6 min at 1,336 $\mu W/cm^2$ per s) vaccinia virus. The cultures were incubated in medium containing 25% concanavalin A-stimulated rat spleen culture supernatant as a source of T-cell growth factor.

At the end of the 7-day culture period, the contents of each well were divided into three portions and assayed on ^{51}Cr-labeled vaccinia virus-infected or normal L cells and YAC-1 target cells. The frequency of responder cell occurrence was determined as the number of cells required to give negative results in 37% of the wells for lysis of a given target as extrapolated from a regression analysis for the plot of cell dilution versus the log percent negative wells. The frequency of virus-specific CTL precursor occurrence was obtained by correcting for nonspecific background lysis on uninfected targets.

Virus plaque assay. The virus plaque assay was performed as described by Ensinger (16). Briefly, monolayers of BSC-40 cells were infected with appropriate dilutions of supernatant from homogenized vaccinia virus-inoculated brain samples. The monolayers were overlaid with medium containing 1% purified agar (Difco Laboratories, Detroit, Mich.) incubated at 37°C for 3 days. The cells were then stained with neutral red dye and reincubated for 1 day, and the number of PFU was determined for each virus dilution.

Measurement of IFN. Serum samples were assayed for the amount of antiviral activity by the microplate method described previously (30), for which encephalomyocarditis virus and monolayers of LF mouse fibroblast cells were used. IFN levels (in units per milliliter) were calculated by comparing the 50% cytopathic effect endpoint titers of experimental samples with those of a known quantity of IFN (alpha and beta) obtained from Lee Biomolecular Research Laboratories (20061; San Diego, Calif.).

RESULTS

Inflammatory cells in CSF of virus-challenged mice. Mice inoculated i.c. with 10^3 PFU of vaccinia virus in 30 μl of PBS developed a progressive nonlethal meningitis, with more than 3×10^5 cells recoverable from the CSF of each animal at the peak of the inflammatory process on day 7 postinjection. This was true for both randomly selected B6 (H-2^b) and (C3D2)F_1 (H-2^{kxd}) strains of mice used for this study. Most of the cells (approximately 70 to 80%) at the latter stages of infection expressed the Thy-1.2 phenotype exhibited by T cells and some subpopulations of NK-like cells (groups A, C, E, and H, Table 1). The expression of the Lyt-1 antigen, which is present on nearly 100% of T cells as detected by fluorescence (33) but not on NK-like cells (28), confirmed the high involvement of T cells in CSF. A significant proportion of the CSF cells also expressed the Lyt-2 marker indicative of the suppressor and cytotoxic subclasses of T cells (either Lyt-1^-2^+ or Lyt-1^+2^+). Administration of 25 μg

TABLE 2. Cytotoxic activity of CSF inflammatory cells from mice injected i.c. with vaccinia virus[a]

Expt	Group	Injection	Anti-Lyt-2 treatment[b]	% Specific ^{51}Cr release on cells[c]:		
				Virus infected	Unin-fected	YAC-1
1	A	PBS	−	77	27	27
			+	34	33	25
	B	Poly(I)-poly(C)	−	80	45	50
			+	34	37	30
2	C	PBS	−	72	14	24
			+	26	NT	28
	D	Poly(I)-poly(C)	−	64	25	44
			+	38	NT	50

[a] The groups correspond to those listed in Table 1.
[b] CSF cells were treated with monoclonal anti-Lyt-2 plus complement before assay. The E/T ratio was adjusted to the remaining viable cells.
[c] Approximate histocompatible L (*H-2^k^*) and MC57-G (*H-2^b^*) target cells were used for experiments 1 and 2, respectively, at an E/T ratio of 30:1. Target cells were either infected with vaccinia virus or uninfected. YAC-1 is an NK cell-sensitive cell line. NT, Not tested.

of poly(I)-poly(C) intravenously (i.v.) 4 and 6 days after virus challenge considerably diminished the magnitude of the inflammatory process compared with the i.v. injection of 0.5 ml of PBS given to mice in the control group. This phenomenon was evidenced by a two- to threefold decrease in the total number of CSF cells and as much as a fourfold decrease in the Thy-1-, Lyt-1-, and Lyt-2-positive T cells (compare group A with B and C with D, Table 1). A similar quantitative decrease was noted when NSS (0.15 ml) was administered i.p. 30 min before injection of poly(I)-poly(C) on day 4 (groups E and F), whereas treatment with sheep anti-mouse IFN antiserum (0.15 ml) prevented this decrease and even seemed to increase the number of cells present in CSF (group G). Neither NSS nor anti-IFN treatment of virus-challenged mice injected with PBS alone on days 4 and 6 postchallenge significantly affected the number of cells obtained from the CSF (groups H through J).

Cytotoxic activity of CSF cells. Table 2 shows the cytotoxic activity of CSF cells quantitated in experiments 1 and 2 of Table 1 and the effects of anti-Lyt-2 antibody plus complement treatment on the response against appropriate vaccinia virus-infected H-2-identical target cells and the NK-susceptible YAC-1 cell line. A large percentage of virus-infected cells were lysed by the CSF from mice inoculated i.c. with PBS only. A significant degree of lysis was apparently due to Lyt-2$^+$ effector cell activity, based on the residual lysis of normal uninfected and YAC-1 target cells after treatment with anti-Lyt-2 plus complement (groups A and C, Table 2). (It should be noted that only viable cells were used to calculate the appropriate effector-to-target cell [E/T] ratios for lysis, so that the Lyt-2$^-$ population had similar activity for all depleted groups.) There was no diminution of the overall cytotoxic response with CSF from poly(I)-poly(C)-treated mice, although the contribution made by NK-like cells seemed to be enhanced (groups B and D, Table 2), as indicated by increased lysis of the YAC-1 target cells.

Frequency analysis. This notion of enhanced NK activity after poly(I)-poly(C) treatment was further substantiated by relative frequency data for cytotoxic precursors in the CSF obtained through limiting-dilution analysis. The poly(I)-poly(C)-treated group F exhibited an approximately ninefold increase in the relative frequency of cells capable of lysing YAC-1 target cells (1:17) over that in control group E (1:159) (Table 3). By taking into account this frequency of effector cell occurrence, the average absolute number of these cells in the CSF of mice can be extrapolated from the total number of cells (Table 1). In this regard, there was an almost threefold increase in the absolute number of NK-like cells in the CSF of mice given poly(I)-poly(C) and NSS (from 3.21×10^4 to 9.41×10^4, Table 3), even though the total cell number had decreased to one-third of the control level (5.1×10^5 to 1.6×10^5, Table 1). Interestingly, the presence of anti-IFN antiserum (group G) did not appear to block this increase in the absolute number of NK-like cells, although the relative frequency (1:105) decreased toward the control level.

On the other hand, the relative frequency of CTL precursors specific for vaccinia virus-infected target cells was virtually unaffected by treatment of the mice with poly(I)-poly(C) (1:168 versus 1:166, Table 3). However, because there was a threefold reduction in the average total number of CSF cells for this group, the absolute number of specific CTL precursors in the CSF reflected a similar decrease (from 3.04×10^4 to 0.96×10^4 cells.) This decrease was reversed by the administration of anti-IFN to poly(I)-poly(C)-treated mice (group G), which actually resulted in an almost 50% increase in the population (4.44×10^4 cells) compared with that in the PBS control, which reflected the near doubling of the total cell number (10×10^5, Table 1), whereas the relative frequency was reduced (1.225). In a separate control experiment (no. 4) NSS treatment apparently had no significant effect on the relative frequency or absolute number of either virus-specific CTL or NK-like cells in the CSF of mice (group I) injected i.v. with PBS on days 4 and 6 after i.c. vaccinia virus challenge. However, the anti-IFN antiserum-treated group J exhibited an approximately 50% increase in the population of both types of effector cells.

IFN levels in serum. To ensure that i.v. administration of poly(I)-poly(C) actually induced high levels of IFN in the serum of vaccinia virus-challenged mice, samples were obtained on day 4 after vaccinia virus infection [1 h before and 6 h after the first injection of poly(I)-poly(C)], on day 6 (6 h after the second injection), and on day 7 (30 h after the second injection). The mice receiving the poly(I)-poly(C) injections had higher systemic levels of IFN than the mice treated with PBS that were sampled at the same times (Fig.

TABLE 3. Relative frequency and absolute numbers of cytotoxic cell precursors in the CSF of mice challenged i.c. with vaccinia virus[a]

Expt	Group	Treatment	Relative frequency (absolute no. × 10^4)[b] specific cytotoxic responder cells to:	
			Virus-infected target cells	YAC-1 cells
3	E	PBS	1:168 (3.04)	1:159 (3.21)
	F	Poly(I)-poly(C) and NSS	1:166 (0.96)	1:17 (9.41)
	G	Poly(I)-poly(C) and IFN-α	1:225 (4.44)	1:105 (9.52)
4	H	PBS	1:212 (1.89)	1.422 (0.95)
	I	PBS and NSS	1:249 (2.37)	1:540 (1.09)
	J	PBS and IFN-α	1:163 (3.07)	1:324 (1.54)

[a] The groups correspond to those listed in Table 1. The CSF cells were stimulated under limiting-dilution conditions by exposure to irradiated spleen cells that were infected with UV-inactivated vaccinia virus. Parallel cultures were assayed 6 days later with vaccinia virus-infected or normal L cell targets or YAC-1 cells.
[b] The relative frequency of CTL specific for virus-infected target cells was obtained by correcting for nonspecific lysis of normal uninfected L target cells. For all calculated frequencies, the correlation coefficient (R) values for the plot of number of cells per well versus the log percent negative wells were greater than 0.95. The absolute number of cytotoxic cells was calculated from the observed relative frequency and the average total number of CSF cells obtained per mouse as shown in Table 1.

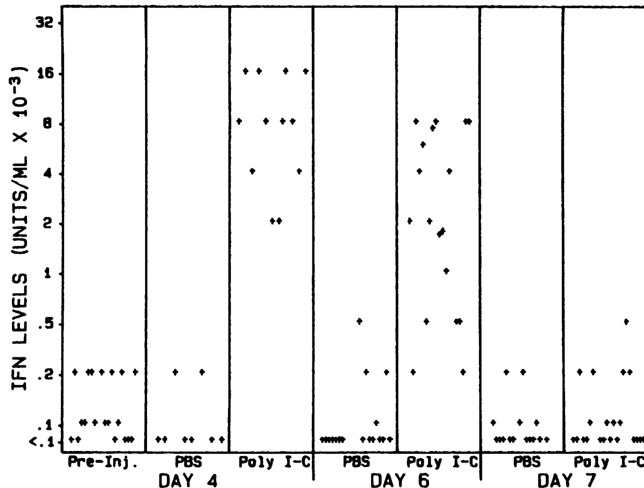

FIG. 1. Interferon levels in serum of mice challenged i.c. with 10^3 PFU of vaccinia virus and treated i.v. with either poly(I)-poly(C) or PBS on days 4 and 6 postinoculation. Samples were obtained on days 4, 6, and 7 (see the text). Results are expressed as individual IFN levels 8 to 21 mice per group.

1). As might be expected, the IFN levels ranged higher after the initial injection on day 4 (2×10^3 to 16×10^3 U/ml) than after the second injection on day 6 (0.2×10^3 to 8×10^3 U/ml.

Infectious vaccinia virus titer in the brain. Taking into account the high IFN levels in serum after poly(I)-poly(C) induction, we questioned whether the observed reduction of the inflammatory process described above was a consequence of direct IFN antiviral activity. Brain samples obtained 6 to 8 days after infection from individual control PBS-treated mice and poly(I)-poly(C)-treated mice were assayed for infectious vaccinia virus. There were no apparent quantitative differences in the numbers of PFU regardless of whether high levels of IFN were induced by poly(I)-poly(C) (Table 4).

DISCUSSION

The model presented in this report for localization of an inflammatory process, particularly that of CTL in the CSF of mice inoculated i.c. with antigen, has been used previously to study the response to lymphocyte choriomeningitis virus (43), ectromelia virus (21), Semliki Forest virus (10), vaccinia virus (11), and tumor cells (14, 15, 31). With vaccinia virus, reasonably large volumes (>20 μl) of CSF could be aspirated from the cisterna magna of mice (11) on day 7 after i.c. inoculation with 10^3 PFU of virus. After this time, the volume of CSF was greatly decreased due to obliteration of the cisterna magna as a consequence of brain swelling. This model has the major advantage of allowing direct examination of the development and nature of a host immune response, as the CSF from normal mice contains very few cells (11, 13–15, 21, 43). In addition, there was minimal contamination with blood during the sampling procedure, and the elevated CSF count resulting from the trauma of i.c. inoculation returns to background levels within 3 to 4 days after diluent rather than virus is injected (10, 13).

The data presented in this report indicate that injection of poly(I)-poly(C) into mice challenged i.c. with vaccinia virus results in a significant reduction in the overall number of cells participating in the central nervous system inflammatory process. There was a fourfold reduction in cells bearing the Thy-1$^+$, Lyt-1$^+$, and Lyt-2$^+$ T-cell markers. Although the relative CTL precursor frequency was unchanged within that reduced population, there was also a significant reduction in the absolute number of specific antiviral effector cells. On the other hand, the relative frequency of NK-like cells (capable of lysing YAC-1 target cells) was greatly enhanced, with a tripling of the absolute number of these cells in the CSF. As a result, there was no observable reduction in the cytotoxic potential of the CSF cells against virus-infected target cells, but this seemed to be due to a shift towards nonspecific NK-like activity rather than to an increased ability of the fewer CTL effector cells to kill their targets. The increased activity of NK-like cells in CSF is consistent with the known effects of IFN (18, 37).

IFN endogenously produced in response to poly(I)-poly(C) injection is strongly implicated as the mediator of the effect on the inflammatory response in this model. This notion is supported by the presence of high levels of IFN in serum after poly(I)-poly(C) administration and the fact that the observed effects on the CTL population specifically and all T cells in general in the CSF were reversed in the presence of anti-IFN antiserum. In fact, the number of T cells appeared to have increased, possibly due to inhibition of IFN released in brain tissue in response to the initial virus challenge. However, antibody treatment did not block the increase in the NK-like cell population in the CSF after poly(I)-poly(C) injection. Perhaps these cells were still being stimulated by residual unbound IFN being continually produced by poly(I)-poly(C)-induced host cells and the gradual depletion of anti-IFN antibodies in vivo. Previous studies have demonstrated the rapidity with which NK cells can be recruited to the CSF in development of the inflammatory response to virus (11).

Interestingly, the poly(I)-poly(C)-induced IFN did not alter the level of viral replication in the brain, so that reduced inflammatory response was not apparently due to reduced viral antigen stimulation of the immune response. The lack of IFN antiviral activity, however, may be due to the fact that excess endogenous IFN was already present, induced by vaccinia virus challenge at the local level in the brain, as vaccinia virus is known to be a potent inducer in its own right (19, 42). This notion is supported by the data in Fig. 1, which show that several of the vaccinia virus-challenged mice had detectable levels of IFN in serum on days 4, 6, and 7. The findings are therefore consistent with the IFN's acting in some manner directly upon the inflammatory cells

TABLE 4. Comparative infectious titer of virus in brains of mice injected i.c. with vaccinia virus[a]

Treatment	Samples obtained (day postinoculation)	Avg no. of PFU ($\log_{10} \bar{x} \pm$ SE)
PBS	6	4.5 ± 0.5
	7	4.3 ± 0.5
	8	3.5 ± 0.3
Poly(I)-poly(C)	6	4.5 ± 0.1
	7	4.7 ± 0.4
	8	4.2 ± 0.4

[a] Adult $(C3D2)F_1$ mice were injected i.c. with a 10^{-4} dilution of vaccinia virus (10^3 PFU). They were then given either PBS or 25 μg of poly(I)-poly(C) i.v. (0.5 ml) on days 4 and 6 postinoculation. Brain samples were taken on days 6, 7, and 8, homogenized in 5 ml of saline containing 0.1% bovine serum albumin, and centrifuged at 3,000 rpm. Dilutions of the supernatant were then used to infect BSC-40 cell monolayers in a virus plaque assay. Results are expressed as the average number of infectious PFU for at least three individual mice per group.

in the CSF (e.g., to retard proliferation) or on the recruitment of cells to the CSF. Certainly IFN has been shown in the past to be capable of regulating the proliferation of T cells in vitro (22, 34, 35) in response to allogeneic stimulation, although sometimes with opposite effects. On the other hand, there is recent evidence that IFN can also affect T-cell circulation (20, 30, 40) in vivo, an obviously important requirement for T-cell recruitment to an antigenic site. Thus, at this time the mechanism of the IFN effect on the inflammatory response is unclear. However, it is clear from the data presented here that when it is considered beneficial to promote virus-specific T-cell function in treating various diseases, the administration of strong IFN inducers or perhaps even of large amounts of exogenous IFN should be approached with caution.

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