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Experimental Allergic Encephalomyelitis in the Rat: Enhancement and Suppression by Synthetic Polyribonucleotides[1] (39631)

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Experimental allergic encephalomyelitis (EAE) is an autoimmune demyelinating disease of the central nervous system (CNS) (1). The disease can be transferred passively with lymph node cells but not with serum (2). EAE is commonly elicited by intracutaneous injection of CNS tissue antigens emulsified in Freund's complete adjuvant (CFA) (3). Recently, complexes of the synthetic polyribonucleotides, polyadenylic acid:polyuridylic acid (A:U) and polyinosinic acid:polycytidylic acid (I:C) have been substituted for mycobacteria in CFA and the resultant adjuvants were used successfully for the induction of EAE in guinea pigs (4, 5). However, polyribonucleotide complexes can often enhance humoral and cellular immunity even when injected separately from antigen and without the other components of CFA (6-17). Therefore, attempts were made here to ascertain whether I:C or A:U could enhance EAE even when administered separately from antigen and as sole adjuvant. The polyribonucleotide complexes were also tested for effects on fully developed EAE and on its passive transfer.

Methods. Lewis male rats (Microbiological Associates, Inc., Walkersville, Md.) weighing 220-380 g were employed. The rats were maintained on Purina Laboratory Chow and water. Some rats were bilaterally adrenalectomized through a mid-dorsal incision and were given saline as sole fluid thereafter.

Double-stranded complexes of I:C and A:U were either purchased from P-L Biochemicals Inc. (Milwaukee, Wis.) or prepared from single stranded polyribonucleotides (Miles Laboratories, Kankakee, Ill.). In the latter case, equal molar concentrations of polyadenylic acid (A) and polyuridylic acid (U) or polyinosinic acid (I) and polycytidylic acid (C) were incubated together for 1 hr at room temperature. The polyribonucleotides were injected into the penile vein (iv) while the rats were under light ether anesthesia.

An intraperitoneal (ip) injection of 100 mg (wet wt) of rat spinal cord homogenate in saline without any adjuvants constituted a weak encephalitogenic challenge (18), whereas severe EAE was elicited by intracutaneous injection (right foot pad) of 0.05 ml of 40% guinea pig spinal cord homogenate emulsified in an equal volume of CFA. The rats were inspected 8 days after challenge and every day thereafter for clinical signs which include flaccidity of tail, and weakness or paralysis of hindlimbs. All rats were killed 15-18 days after challenge. The entire spinal cords were fixed in Bouin's fluid, embedded longitudinally in paraffin, stained by hematoxylin and eosin, randomized, and scored for histologic EAE lesions.

Passive transfer of EAE was accomplished by injecting rats iv with "EAE cells," obtained from lymph nodes draining sites of inoculation in donors that had been immunized 8 days previously in the right foot pad with 0.05 ml of guinea pig spinal cord homogenate emulsified in CFA (19). A donor:recipient ratio of 1:16 constituted a weak challenge whereas a ratio of 1:1 or 1:2 was a strong challenge. The rats were examined for clinical signs daily. When it was desirable to elicit histological EAE lesions within 24 hr after the injection of EAE cells, a localized form of passive transfer was employed. Acceleration and localization of the EAE lesions was induced by inflicting a thermal injury in the right cerebral hemisphere of the recipients 2 days before the transfer (19). The rats were sacrificed 1 day after the cell transfer. The brains were fixed, embedded, sectioned coronally,

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and stained as above, and the slides were randomized and scored for EAE lesions localized near the region of the thermal necrosis.

Results. One iv injection of 0.6 mg of I:C, administered at the time of a weak encephalitogenic challenge, resulted in a moderate but significant increase ($P < 0.05$) in the number of rats that had clinical and histological manifestations of EAE, whereas administration of 0.6 mg of A:U or of I alone or C alone had no effect (Table I). I:C treated rats also had more extensive histological lesions than controls; this difference barely failed to reach statistical significance when only animals with lesions were analyzed ($P > 0.05$), but it was significant when all rats were included ($P < 0.01$). The adjuvant effects of I:C were only observed when the encephalitogenic stimulus was weak (i.e., 100 mg of rat spinal cord ip); pilot experiments have shown that an increase of the challenge dose to 200 mg of cord increased the incidence and severity of EAE and administration of I:C did not cause further enhancement.

The mechanism by which I:C enhances immunization against a weak encephalitogenic stimulus was investigated with the aid of the passive transfer system. The ability of polyribonucleotides to affect the activity of fully immunized EAE cells in the rapid (1-day) localized passive transfer system was studied first. The results were the opposite of those obtained with active immunization. I:C, administered to recipients on the day of cell transfer, greatly reduced the numbers of localized EAE lesions if the donor:recipient ratio was 1:1 and completely prevented the formation of localized lesions if the donor:recipient ratio was 1:16 (Table II). Both Miles Laboratories and P-L Biochemicals preparations of I:C were effective. The suppression of localized EAE was not due to nonspecific adrenal-mediated stress effects inasmuch as I:C was also inhibitory in adrenalectomized recipient rats. Since I:C is quite toxic for adrenalectomized rats (20), only small quantities of I:C (0.06 or 0.3 mg) were used in this experiment. These small doses were equally effective in intact and adrenalectomized recipients (Table II). Other ribonucleotides were not effective; A:U on the day of EAE transfer was much less suppressive than I:C, and I alone or C alone had no effect. The I:C effects were transient; the polyribonucleotide complex lost much of its suppressive property when administered 1 day before the cell transfer and was not effective at all when administered 6 days before the transfer.

TABLE I. EFFECTS OF SYNTHETIC POLYRIBONUCLEOTIDES ON THE INCIDENCE AND SEVERITY OF EAE IN LEWIS RATS CHALLENGED IP WITH RAT CORD HOMOGENATE.

Treatment on day of challenge	Incidence of clinical signs[a]	Incidence of histological lesions[a]	Average severity of histological lesions[b]
I:C	14/52	29/51	2.9
A:U	1/28	8/28	1.7
I	1/14	4/14	2.1
C	0/14	4/14	1.0
Saline	2/27	8/27	2.1

[a] Numerator: number of rats with clinical signs or histological lesions. Denominator: total number of rats.

[b] Scored from 1+ to 4+ according to number and extent of perivascular inflammatory lesions in sections of entire spinal cord. Calculation of averages did not include negative specimens.

The possibility was considered that suppression of localized EAE by I:C was not a direct function of the polyribonucleotide complex but might have been due to induction of interferon. Interferon can reach high levels in rat blood within several hours after I:C administration (21) and it may be immunosuppressive (22). Aliquots of 4.0 ml of pooled blood or sera obtained from rats that had been injected iv with 1.2 mg of I:C 2 hr earlier were injected into recipient rats immediately after EAE cells (donor:recipient ratio of 1:1). These bloods and sera were strongly inhibitory to EAE (Table III). However, prior incubation of 4.0-ml aliquots of the sera for 1 hr at 37° with 0.96 mg of ribonuclease A (RNase, from bovine pancreas, Sigma Chemical Co., St. Louis, Mo.), eliminated the inhibitory properties of the sera. A control incubation mixture composed of RNase and normal rat serum did not enhance or suppress the development of passively transferred, localized EAE. Hence, the inhibitory properties of blood and sera from I:C-treated rats were due to their residual content of the RNase-susceptible polyribonucleotide complex and were not due to interferon.

It was important to establish whether I:C

TABLE II. EFFECTS OF SYNTHETIC POLYRIBONUCLEOTIDES ON PASSIVELY TRANSFERRED LOCALIZED EAE.

Recipients and donor:recipient	Treatment of recipients	Time[a]	Histologic scores (individual rats)[b]
Intact 1:1	I:C, 0.6 mg	DO	1 1 1 1 1 1 1 0 0 0 0 0
		D-1	4 3 1
		D-6	4 4 4
	A:U, 0.6 mg	DO	4 3 3 3 3 3
	I, 0.6 mg	DO	4 4 4 4 4 4
	C, 0.6 mg	DO	4 4 4 4
	Saline (control)	DO	4 4 4 4 4
Intact 1:16	I:C, 0.6 mg	DO	0 0 0
		D-1	1 0 0
		D-6	2 1 1
	Saline (control)	DO	2 1 1
Adrenex[c] 1:2	I:C, 0.3 mg	DO	2 2 2
Intact 1:2		DO	2 1 0
Adrenex 1:2	I:C, 0.06 mg	DO	2 2 1 0 0 0 0
Intact 1:2		DO	2 2 2
Adrenex 1:2	Saline (control)	DO	4 3 3 3
Intact 1:2		DO	4 4 3

[a] DO = day of passive transfer of EAE cells. D-6 and D-1 refer to 6 and 1 days before passive transfer.
[b] Scored from 0 to 4+ according to number and extent of perivascular inflammatory lesions localized adjacent to the zone of thermal brain necrosis.
[c] Bilateral adrenalectomy 3 days before cell transfer.

TABLE III. EFFECTS OF I:C-CONTAINING BLOOD AND SERUM ON PASSIVELY TRANSFERRED, LOCALIZED EAE.

Treatment of recipients[a]	Histologic scores (individual rats)[b]
Saline	4, 4, 4, 4, 4, 3
Blood from I:C-treated rats	1, 0, 0, 0, 0, 0, 0
Serum from I:C-treated rats; serum incubated with saline	0, 0, 0, 0
Serum from I:C-treated rats; serum incubated with RNase	4, 4, 4, 3
Serum from untreated rats; serum incubated with RNase	4, 4, 4, 3

[a] All recipients received EAE cells at a donor:recipient ratio of 1:1. All treatments were 4 ml in volume.
[b] Scored from 0 to 4+ according to number and extent of perivascular inflammatory lesions localized adjacent to the zone of thermal brain necrosis.

permanently destroyed or only temporarily suppressed the activities of fully immunized EAE cells. Therefore, I:C was tested in the nonlocalized passive transfer system where there is a 4-day latent period between administration of EAE cells and the development of clinical signs. I:C, 0.6 mg, was administered iv to recipients 1 day after they had received the passive transfer of EAE cells. The disease was not significantly altered; the four I:C-treated recipients developed clinical signs 5 or 6 days after passive transfer and they all became paralyzed, while the four saline-treated controls developed signs 4 to 6 days after transfer and two became paralyzed and two became weak. This indicates that the suppressive effects of I:C on the fully immunized EAE cells were transient and nondestructive.

In spite of the transient nature of the inhibition of fully immunized EAE cells by I:C, we hoped that these short-term effects could be utilized to treat EAE after clinical signs had appeared. EAE was, therefore, produced in 21 rats by active immunization with guinea pig spinal cord and CFA in the footpad. At the time that clinical symptoms first appeared (11 days after challenge), the rats were divided into three equal groups which were treated iv once with 0.6 mg of I:C or with A:U or with saline. None of the treatments changed the course of the illness. In each group there were five or six deaths after nearly equal survival periods (3.0, 3.4, 2.7 days, average, respectively) after treatment. Apparently, the effects of I:C were too weak or too transitory to have any effect at such a late stage of the disease process.

Discussion. Gumbiner *et al.* (4) and Paterson (5) have demonstrated that guinea pigs injected intracutaneously with guinea pig spinal cord and Freund's adjuvant developed EAE if either mycobacterial RNA, A:U, or I:C were included in the emulsion. Paterson (23) speculated that the adjuvant effects of these double-stranded polyribonu-

cleotides rested on their ability to activate macrophages and to expand the numbers of specific thymus-derived lymphocytes which can be sensitized by myelin basic protein.

The present experiments differed from Gumbiner *et al.* (4) and Paterson (5, 23) in that we used rats instead of guinea pigs, an aqueous encephalitogenic antigen without adjuvants instead of an emulsion in CFA, and a separate iv injection of polyribonucleotide instead of an admixture with antigen. Nevertheless, we also observed enhancement with I:C, but unlike these authors, there was no enhancement with A:U. Recently, Paterson (24) reported that even in the absence of CNS antigens, Freund's incomplete adjuvant containing either A:U or A was able to induce EAE lesions in the choroid plexus and neuraxis of guinea pigs. There is no evidence that I:C can elicit this phenomenon or that it can occur in rats. Nevertheless, we verified the nonencephalitogenicity of I:C under the conditions of these experiments by injecting 0.6 mg iv into each of five rats and observing them for 17 days. There were no clinical signs or histologic lesions of EAE.

A number of experimental designs have demonstrated that I:C and A:U can enhance cellular immunity and delayed hypersensitivity (4, 5, 10, 12–17). However, in other experimental designs, I:C has been shown to suppress cell mediated immune responses (11, 25–28). As far as we know, the present study is the first one to demonstrate the ability of I:C to enhance and to suppress, respectively, the initiation and the expression of the same delayed hypersensitivity syndrome. Enhancement of cell-mediated immune responses by I:C has often been attributed to the ability of I:C to increase the proliferation of thymus-derived lymphocytes (4, 5, 17) and/or to stimulation or activation of macrophages (5, 14–17). Suppression of cell-mediated immune responses by I:C has been blamed on its cytotoxicity (11, 25–28); I:C can cause a leukopenia and lymphopenia in the peripheral circulation (29, 30); depletion of lymphoid tissues has also been described (31). In the present study, too, depletion of lymphocytes from thymus-dependent areas of the spleen occurred regularly in recipients of a localized passive transfer, 1 day after administration of I:C. Furthermore, I:C also causes a transient suppression of phagocytosis in the reticuloendothelial system (RES) which is followed within several days by a hyperactivity of RES functions (32, 33). Lymphopenia and transient suppression of leukocyte activities could explain the short-lived suppression of passively transferred EAE since development of EAE depends both on donor and recipient's leukocytes (1). Rebound hyperactivity of RES and lymphoid tissues functions at a later date might increase the ability of certain cells (i.e., macrophages and thymus-derived lymphocytes) to respond to neural antigens, and may thereby enhance the development of EAE in actively sensitized rats. If these explanations are correct, one would expect A:U which is much less toxic than I:C (34) to have a smaller effect on EAE. This expectation was fulfilled.

Transient toxic effects of I:C on leukocytes and lymphocytes and subsequent activation of macrophages and proliferation of lymphocytes could explain the apparent contradictions between a number of reports some of which have shown I:C to enhance (4, 5, 10, 12–17) while others have shown I:C to suppress (11, 25–28) cell-mediated immune responses.

Summary. Lewis rats injected ip with 100 mg of rat cord homogenate developed a mild degree of experimental allergic encephalomyelitis (EAE). One iv injection of 0.6 mg of the polyribonucleotide complex, polyinosinic acid:polycytidylic acid (I:C), administered at the time of encephalitogenic challenge, increased the incidence and severity of EAE whereas polyadenylic acid: polyuridylic acid (A:U) and the single-stranded polyinosinic acid (I) or polycytidylic acid (C) had no effect. Injection of I:C at the time of onset of clinical signs of EAE did not influence the course of the illness.

The mechanism of enhancement of EAE by I:C was investigated with the aid of the passive transfer system. Unexpectedly, I:C inhibited the development of EAE lesions when injected into recipients along with the fully immunized cells from donors with EAE. The inhibitory effect of I:C on EAE cells was transient and nondestructive. Inhi-

bition by I:C was not mediated by the adrenal gland or by induction of interferon. The apparently opposite effects of I:C on active and passive production of EAE may be explained by its biphasic effects on lymphocytes and reticuloendothelial function.

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