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Suppression of the Mixed Leukocyte Reaction by Serum from Polynucleotide-Injected Mice (42130)

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Abstract. Sera from mice which have been injected iv with either poly(A)·poly(U) or poly(I)·poly(C) 1½ hr prior to bleeding were found to suppress the mixed lymphocyte reaction. This effect was reduced considerably by 18 hr. Characterization of the suppressive sera revealed it (a) was stable to heating at 56°C for 1 hr and freezing at −20°C for 1 month; (b) had a molecular weight greater than 30,000; (c) could be induced in sera from athymic nude mice; and (d) was present to a lower degree in sera from aging mice.

The synthetic polynucleotide double stranded complexes, polyadenylic–polyuridylic acid and polyinosinic–polycytidylic acid have been shown to be very effective immunomodulating agents (1). Thus, injection of either complex with antigen results in a marked stimulation of antibody synthesis. Contrariwise, injection of the complex 1–2 days before antigen results in substantial inhibition of humoral immunity (1). In recent experiments designed to determine the mechanism by which such synthetic polynucleotides inhibit immunity, a rapidly appearing suppressive action on the mixed leukocyte reaction was detected in the serum of polynucleotide-injected mice. Characterization of such suppressive sera is described herein.

Materials and Methods. *Mixed lymphocyte reaction (MLR).* Washed, single cell suspensions were made from spleens removed from Balb/c (responder) and C58 (stimulator) mice and resuspended in 5 ml of H-3 culture medium, counted and diluted to 1×10^7 cells/ml. The cells designated to be stimulator cells and a small volume of responder cells used as controls were each mixed with sterile mitomycin C (1 ml diluted cells per 0.5 ml mitomycin C at 0.08 mg/ml) and placed in a shaking water bath at 37°C for 45 min. The cells then were centrifuged at 300*g* for 10 min, resuspended in approximately 10 ml HBSS, and washed two times. They were resuspended in the required volume of H-3 medium, counted and diluted to 1×10^7 cells/ml with H3 medium. In the MLR, six wells of a round bottom microtiter plate were treated identically for each experimental variable. Control wells contained 0.05 ml of mitomycin-treated responder cells (Balb/cm) diluted to 1×10^7 cells/ml and 0.05 ml of similarly diluted responder cells. The MLR wells contained 0.05 ml of mitomycin-treated stimulator cells (C58m) and 0.05 ml of responder cells. The experimental groups had 0.05 ml of C58m cells and 0.05 ml Balb/c cells added to the wells, plus either 10 μl of control (PBS-injected mice) or adjuvant-induced sera. The volume in each reaction well was brought up to 0.2 ml by addition of 0.1 ml H-3 culture medium or this medium containing the experimental adjuvants. The plates were incubated at 38°C for 2 days at which time 1 μCi of [^{3}H]thymidine (Schwartz-Mann, Orangeburg, N.Y.) was added to each well in 0.05 ml. Approximately 18 hr later, the cells from each well were harvested onto glass-fiber filters, dried, and placed in a scintillation vial with 5 ml PPO–POPOP–toluene scintillation fluid. Each vial was counted twice for 5 min, the average of the two counts determined, and the mean counts per minute and standard error calculated for each experimental group.

Preparation of control (PBS) and adjuvant-induced sera. Mice were injected iv with either PBS or 300 μg of one of the following adjuvants: poly(A)·poly(U), poly(I)·poly(C), or muramyl dipeptide (MDP). The mice were anesthetized 1½ or 18 hr later with ether and bled from the axillary fossa or by cardiac puncture. Blood was pooled from multiple

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mice, allowed to clot 1 hr or longer on ice and the serum recovered after centrifugation at 1700 rpm for 15 min at 4°C. The serum was filter sterilized and added to the MLR reaction well as indicated.

Interferon assay. Interferon in serum was measured by the hemagglutination yield reduction bioassay (2) utilizing L929 murine cells and GD VII virus. This assay measures the same unitage (12,000) assigned to the mouse interferon-α/β International Reference Preparation G. Titers are expressed as international units (IU/ml). Neutralization tests were carried out against 8–10 units of interferon, using NIH G024-501-568 antiserum to MuIFN-α/β, and highly specific antiserum to MuIFN-β from Lee Biochemicals (San Diego, Calif.) and to MuIFN-γ (from Dr. E. Havell, Saranac Lake, N.Y.).

Animals. Balb/c mice of either sex were used for serum preparation and for responder cells in the MLR. Both the Balb and C58 mice were raised in our animal facilities and were used at 7–12 weeks (young) or 65–95 weeks (aging). Male, 8-week-old, athymic nude mice were obtained from Charles River Laboratories, Wilmington, Massachusetts.

Adjuvants. Poly(A)·poly(U) (Miles Scientific, Naperville, Ill.) and poly(I)·poly(C) (Calbiochem-Behring, San Diego, Calif.) were prepared by annealing equal volumes of poly(A) and poly(U) each at 6 mg/ml and poly(I) and poly(C) each at 3 mg/ml for 30 min at 37°C, after which appropriate dilutions were made with phosphate buffered saline, pH 7.2. MDP was a gift from Dr. Louis Chedid, Institut Pasteur, Paris, France. All dilutions were made in PBS and injections given iv.

Statistical analysis. Data were analyzed by a BMDP statistical program for a one-sample t test after taking the logarithm$_{10}$ of the ratio of each pair of groups compared.

Results. Suppression of the MLR (Balb × C58m) following addition to the reaction well of murine sera removed $1\frac{1}{2}$ hr after iv injection of either of the polynucleotide complexes is summarized in Table I. Both poly(I)·poly(C)- and poly(A)·poly(U)-induced sera suppressed the MLR strongly. The suppression induced by poly(A)·poly(U) in sera collected at 18 hr postinjection had waned to only one-half that exerted by sera collected $1\frac{1}{2}$ hr postinjection (Table I). Of interest was the finding that MDP induced sera removed at $1\frac{1}{2}$ hr did not result in MLR suppression in four experiments (data not shown).

The suppressive effect of the sera on the MLR could not be maintained beyond a 1:10 dilution (Table I). In addition in individual experiments it was observed that control sera from normal mice would either enhance or suppress the cpm relative to wells with no serum added. Although control sera (n = 26) averaged 14% lower than wells with no serum added, this difference was not statistically significant ($P > 0.15$).

To test the possibility that the adjuvant-induced suppressive activity might be a result of tissue damage since blood was collected from the axillary fossa, sera from blood col-

TABLE I. CHARACTERISTICS OF POLYNUCLEOTIDE COMPLEX-INDUCED MLR SUPPRESSIVE SERA

Serum obtained at (hr)[a]	Treatment	Average % suppression[b] induced by	
		Poly(A)·poly(U)	Poly(I)·poly(C)
1.5	—	58 (8)[c]	86 (10)
18	—	26 (6)	N.D.
1.5	1:5 dilution	36 (3)	71 (3)
1.5	1:10 dilution	12 (3)	34 (3)
1.5	Cardiac bleeding	70 (2)	78 (3)
1.5	Heat (56°C)	56 (4)	60 (4)
1.5	Freeze–thaw (−20°C)	69 (6)	85 (5)

[a] 300 μg polynucleotide complex injected iv into 7 to 12-week-old Balb mice.
[b] % Suppression relative to control sera added to MLR.
[c] Number of experiments in parentheses.

lected by cardiac puncture was also tested. Suppression was maintained equally well in such samples (Table I).

To determine whether poly(A)·poly(U) had been retained in the sera and was responsible for the suppression, this adjuvant was added over a wide range of doses from 0.01 to 100 μg to the MLR and had no significant effect.

Preliminary characterization of putative suppressive factors present in the adjuvant-induced sera also is summarized in Table I. The results of four different experiments in which control and various adjuvant-induced sera were heated at 56°C for 45–60 min revealed MLR suppression to be maintained after heating, although there was a slight but significant decrease in the heated poly(I)·poly(C)-induced sera. Suppression also was maintained after freezing at −20°C for up to 1 month.

The molecular size of the suppressive factor was also investigated through the use of Amicon Centricon membranes with 30,000- and 10,000-Da cutoffs. In each of two experiments, the suppression appeared only in the greater than 30,000-Da fraction with both the poly(A)·poly(U) and poly(I)·poly(C)-induced sera. Data from one experiment appear in Table II.

Since interferon has been shown to be immunosuppressive (3) and the heat stability of the suppressive factor(s) was compatible with that established for interferon, sera shown to be suppressive were tested for the presence of interferon. The results (Table III) showed that the PBS sera did not have detectable levels of interferon, while marked differences were seen among the adjuvant-induced sera in that poly(A)·poly(U) and LPS had 200–400 units and poly(I)·poly(C) sera had 16,000 units. When sera from one experiment were assayed with respect to interferon type, poly(A)·poly(U)-induced sera exhibited interferon-α while the other adjuvant-induced sera were positive for interferon-β. In no case was interferon-γ detected.

TABLE II. FRACTIONATION OF ADJUVANT-INDUCED MLR SUPPRESSIVE SERA

Control serum fractions	% of MLR	AU serum fractions	% of MLR
Unfractionated	130	unfractionated	40*
>30,000	125	>30,000	58*
10,000–30,000	107	10,000–30,000	105
<10,000	121	<10,000	112

Note. MLR: Balb + C58m = 12,690 cpm.
* P = <0.01 as compared to control serum fractionation.

Since soluble suppressive factors have been reported to be released from T cells, attempts were made to generate the MLR suppressing factor(s) in the serum of homozygous athymic nude mice. Poly(A)·poly(U)- and poly(I)·poly(C)-induced sera from nude mice gave MLR suppression equivalent to that observed in normal mice (Table IV). Control sera from nude mice injected with PBS were not suppressive.

The decreased ability of aging animals to respond immunologically is well known (4). To test whether this might be due in part to the proficiency at which aging mice might generate MLR suppressive factors, blood was collected from 65- to 95-week-old Balb/c mice $1\frac{1}{2}$ or 18 hr following poly(A)·poly(U) injection. Using young Balb/c spleen cells as responding cells, the cpm resulting from the addition to the MLR of $1\frac{1}{2}$ hr sera from young and aging mice in three experiments averaged 69 and 42% suppression, respectively, as compared to the control sera. By 18 hr this suppression has decreased to 28% for young and 0% for aging mice.

Discussion. The experiments described herein were initiated to explore the mechanism by which polynucleotide complexes inhibit the immune response nonspecifically when injected 18–48 hr before antigen (1). In an earlier study, such suppression was associated with nylon-wool adherent splenic T cells removed 1 day after administration of poly(A)·poly(U) (5). To determine in the present study whether such suppressor cells functioned via a secretory product with a serum phase, serum was collected at 18 hr (a time when antigen encounters a strong inhibitory environment *in vivo*) and tested for any suppressive activity. Serum was also removed $1\frac{1}{2}$ hr after polynucleotide injection

TABLE III. INTERFERON CONTENT OF POLYNUCLEOTIDE-INDUCED SUPPRESSIVE SERA

Serum[a] induced by	% MLR suppression	Interferon IU/ml	Interferon type		
			α	β	γ
PBS (5)[b]	0	<50	−	−	−
Poly(A)·poly(U) (4) (300 μg)	52 ± 9	239 ± 122	++	±	−
Poly(I)·poly(C) (4) (300 μg)	85 ± 2	16,250 ± 3,570	±	++	−
LPS (2) (20 μg)	51 ± 3	450 ± 175	±	++	−

[a] Sera obtained $1\frac{1}{2}$ hr after iv injection.

[b] Number of serum samples assayed are in parentheses.

(a time when injection of antigen results in enhancement of antibody synthesis) to serve as a putative negative control. However, as can be seen in Table I, paradoxically a profound inhibitory action on the MLR, a model of cell-mediated immunity, appeared rapidly in the 90-min sample which was considerably reduced in the 18-hr sample. Thus, this synthetic immunomodulating agent induced a suppressive environment for the CMI branch of immunity at a time ($1\frac{1}{2}$ hr) when an expansive environment exists for the other branch, humoral immunity. These circumstances invite speculation as to whether a suppressed CMI contributes to enhanced antibody synthesis.

A possible T-cell origin of the MLR suppressive factors in serum appears negated by the data in Table IV. Suppression was readily generated by the polynucleotide complexes in athymic mice. In addition, any relationship of such factor(s) to the recently described (6) Il-2 inhibitor present in normal murine sera can most likely be discounted by the requirement for thymus cells to express the Il-2 inhibitor.

TABLE IV. POLYNUCLEOTIDE-INDUCED SERA FROM ATHYMIC NUDE MICE SUPPRESS THE MLR

Mice	% Suppression of MLR[b]	
	Poly(A)·poly(U) sera	Poly(I)·poly(C) sera
Euthymic	40[a]	78[a]
Athymic	43[a]	82[a]

[a] $P < 0.02$ as compared to control sera.

[b] Relative to control sera MLR = Balb + C58m = 10,425 cpm.

Since macrophages have been shown to be activated by synthetic polynucleotides (7), logical candidates for the suppressive factor(s) include the prostaglandins. However, suppression was clearly associated only with that serum fraction greater than 30,000 Da. Interferons-α and -β also activate macrophages (8). The data presented do not exclude mouse interferon-β as one possible mediator in the observed suppression, since its molecular weight is about 35,000, whereas that of MuIFN-α is about 20,000.

The complexity of the host response to the polynucleotide complexes is illustrated when one compares the results of Morris and Johnson (5) to the data herein. In the previous study suppression of *PFC* was attributable to a nylon-wool adherent T-suppressor cell. This appears to contrast with our present finding of suppressive sera being readily induced by polynucleotides in nu/nu mice. The rapidity of the latter effect renders it unlikely as resulting from stimulation of pre-T cells. However, suppression of the humoral response may be mediated by cells and factors different from those effective on the CMI. Further experimentation is needed.

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