THE MITOGENIC ACTIVITY OF POLYADENYLIC-POLYURIDYLIC ACID COMPLEXES

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The capacity of synthetic polyadenylic and polyuridylic acid complexes (poly A:U) to increase antibody synthesis to a number of different antigens given via several different routes has been characterized.[1, 2] The adjuvant activity was most readily apparent when immunity was partially depressed, as for example by thymectomy,[3] or when the antigenicity of the material was low.[1] The functional activities of several different cell types, including lymphocytes, macrophages, stem cells, and memory cells,[4] as well as the immunologically unrelated cells found in the parotid and pancreas,[5] have been shown to be enhanced. The mode of action of the polynucleotides is unknown, but their ability to activate 10^4 T-cells to a state functionally similar to that expressed by 10^6 T-cells not exposed to poly A:U is impressive, and suggests that this adjuvant may amplify the number of T-cells.[3] In addition, poly-A:U-treated mice were found to be susceptible to the antimitotic drug vinblastine some 6 hours earlier than controls, suggesting that cells were stimulated into division more rapidly under the stimulus of poly A:U.[6] To test this hypothesis, the effect of poly A:U on the division of thymocytes, spleen and bone marrow cells, *in vitro* and *in vivo*, was measured and the results are presented herein.

Materials and Methods

Animals

Balb/Aj mice either 4–5 weeks or 10–13 weeks in age were inbred in our laboratory and used throughout the study. They were supplied with Purina rat chow and water *ad libitum*.

Antigen

Sheep red blood cells (SRBC) were obtained from the Colorado Serum Company, Denver, Colorado. The cells were stored at 4° C in modified Alsever's solution, washed 3 times and resuspended in phosphate buffered saline (PBS), pH 7.2, for injection. For *in vivo* experiments 2×10^7 SRBC were injected intravenously, whereas 1×10^6 SRBC was added to each culture dish for experiments *in vitro*.

Homoribopolynucleotides

Polyadenylic acid (poly A), potassium salt (10t 69) and polyuridylic acid (poly U), ammonium salt (lot 77) were purchased from Miles Laboratories,

Elkhart, Indiana. Polymers were complexed *in vitro* to form poly A:U by mixing equal amounts of polynucleotides before use, as previously described.[1]

Cortisone Administration

In experiments aimed at studying the responsiveness of cortisone-resistant spleen cells, 2.5 mg of hydrocortisone sodium succinate [7] were injected intraperitoneally 48 hours prior to the injection of antigen, or poly A:U and antigen. The control mice received appropriate amounts of saline in lieu of cortisone.

In Vitro *Cell Culture*

Spleens and mesenteric lymph nodes from normal, adult Balb mice or thymuses from 4-week-old Balb mice were removed aseptically. Single-cell suspensions were prepared in the following way. Intact organs were washed 2 times with Hank's balanced salt solution (HBSS) containing 100 units of penicillin and 100 μg streptomycin/ml, gently teased, and kept in an ice bath for 10 minutes to permit large fragments to settle by gravity. Suspensions of bone marrow cells were prepared by flushing the marrow from femurs and tibia with a syringe and 26 gauge needle containing cold Eagle's basal medium. The extruded plugs were dispersed by aspiration with a 21 gauge needle and syringe. The fragment-free supernatant was then centrifuged in the cold for 10 minutes at 1,000 rpm. The cell pellet was washed with HBSS and finally suspended into a culture medium containing 100 units of penicillin and 100 μg streptomycin per ml of medium. The concentration of the cell suspension was adjusted to 5×10^6 per ml.

Culture Medium

The culture medium used here consisted of: Aqua dist. water, 85 ml; Hank's MEM 10x, 10 ml; Vitamins 100x, 1 ml; Essential amino acid 50x, 2 ml; Non-essential amino acid 100x, 1 ml; L-glutamine 100x, 1 ml; Pyruvate 100x, 1 ml; Na_2HCO_3 (7.5%), 3.9 ml; and Fetal calf serum, 10 ml.

Incorporation of [^{3}H]Thymidine

Tritiated thymidine (methyl-^{3}H, Code TRK 120 Batch 70, specific activity 18.9 Ci/mM, Amersham/Searle) was used in all *in vivo* experiments at 0.5 μci per gram body weight. For *in vitro* work, 4–6 μci of [^{3}H]thymidine was added to the culture fluid per dish. In early *in vitro* experiments, a labeling period of 24 hours was adopted, whereas in later experiments an incorporation time of 2 hours was used.

Determination of Radioactivity

At the time of sacrifice, mice were killed by cervical dislocation, and the spleen was removed and homogenized with a Harlbrook hand homogenizer in

5 ml of PBS at pH 7.2. One hundred, 200 or 500 μl of homogenate was put on a Millipore® or glass-fiber filter paper, and washed with 10 ml saline, 10 ml of ice cold 10% TCA, 2 ml of 95% ethanol, and 2 ml of absolute ethanol. Following this, the filter paper was transferred to a scintillation vial and 10 ml of scintillation fluid (15 g of PPO and 0.4 g POPOP per gallon of toluene) was added to the sample. The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3320. In light of the variation in total counts among different experiments, results were expressed as percentage of control values ± standard deviations.

Results

Thymus Lymphocytes

Single-cell suspensions of thymus tissue could not be stimulated into division with poly A:U alone under our conditions. Thus, titration of the dose of adjuvant from 1×10^{-4} to 20 μg did not significantly increase the uptake of tritiated thymidine added to separate cultures at either 0, 3, 6, 12, 18, 24, or 48 hours. However, slight, but consistent stimulation was achieved by 5×10^{-2} to 5×10^{-3} μg poly A:U, but only when SRBC was added as antigen (125–130% of controls receiving SRBC alone).

Peripheral T-lymphocytes

Inasmuch as thymocytes per se appeared unable to respond to poly A:U under our culture conditions, the mitogenic effect of poly A:U on the division

Table 1

Kinetics of Mitogenic Effects of Poly A:U and/or SRBC on Mouse Spleen Cells *in Vitro*

Spleen Cells * Incubated with:		[^{3}H]thymidine Incorporation After Culture for Cpm±SD (% cpm) †			
SRBC	Poly A:U	6 hours	12 hours	24 hours	48 hours
−	−	5,600±270 (100±5)	2,700±550 (100±20)	1,580±234 (100±14)	2,000±360 (100±18)
+	−	5,300±700 (93±13)	2,400±150 (88±6)	2,180±192 (138±8)	2,480±225 (124±9)
−	+	4,500±380 (79±8)	3,500±500 (128±14)	1,960±124 (124±6)	2,560±500 (128±5)
+	+	4,300±640 (75±14)	3,300±640 (119±19)	2,400±490 (150±20)	2,440±290 (122±12)

* 5×10^6 spleen cells in 0.5 ml MEM were incubated with 30 μl of 0.1% SRBC (1×10^6) and/or 1 μg poly A:U for different time intervals, followed by the addition of 6 μci [^{3}H]thymidine into the culture fluid and an additional 6 hours incubation.

† (Cpm exp. group/cpm control group) × 100 ± standard deviation.

TABLE 2

In Vitro INCORPORATION OF [^{3}H]THYMIDINE BY NORMAL SPLEEN CELLS IN SERUM-FREE MEDIUM

Spleen Cells * Incubated with: SRBC	Poly A:U	[^{3}H]Thymidine Incorporation for Cpm (% cpm) † ±SD	
−	−	11,600±60	(100±0.5)
+	−	14,900±1200	(130±8)
−	1.5 μg	18,000±800	(155±5)
−	4.5 μg	20,800±960	(180±4)
+	1.5 μg	22,700±4200	(195±18)
+	4.5 μg	26,300±500	(226±2)

* 5×10^6 spleen cells in 1 ml MEM were incubated with 30 μl of 0.1% SRBC (1×10^6) and/or 1.5 μg–4.5 μg poly A:U for 48 hours, followed by 4 μci of [^{3}H] thymidine added into the culture fluid and an additional 18 hours incubation.

† (Cpm exp. group/cpm control group) × 100 ± standard deviation.

of peripheral T-lymphocytes in the spleen was tested. Thus, 5×10^6 spleen cells were cultured for varying periods of time with or without SRBC and/or 1 μg poly A:U/dish. TABLE 1 records the results of one experiment representative of the 6 performed. Beginning at 12 hours of culture, there was an increase in [^{3}H]thymidine incorporation in tissues containing poly A:U both in the presence and absence of SRBC. However, the increase at this time was variable and statistically insignificant. By 24 hours the cells given antigen alone showed a 38% increase in [^{3}H]thymidine incorporation, whereas poly A:U alone produced a 24% increase. Under the combined stimulation of poly A:U and antigen, incorporation increased to 150% of the control value. After 48 hours of culture, incorporation was greater than the control in all experimental combinations, i.e. those containing either the antigen alone, poly A:U alone, or antigen plus poly A:U. However, no significant differences were noted among the 3 experimental groups.

Similar results were obtained when the same experiments were performed without the addition of calf serum. As shown in TABLE 2, the thymidine incorporation by spleen cells in the presence of 4.5 μg poly A:U with or without SRBC was 226% and 180% of the control, respectively. A significant point to be noted was that poly A:U alone, in the absence of serum or antigen, was as mitogenic as SRBC.

In Vivo *Effects of Poly A:U*

In order to place in perspective the results gained in the *in vitro* experiments performed above, the mitogenic effect of poly A:U on spleen cells was tested by giving poly A:U intravenously to mice on day 0 with or without a concomitant injection of SRBC. At 2 hours prior to sacrifice, [^{3}H]thymidine was injected. Results from a representative experiment are recorded in TABLE 3. No significant effect was evident 18 hours after poly A:U administration;

however, 2 days after poly A:U was given, 260% stimulation was observed. Similar values were obtained in animals receiving poly A:U plus SRBC, and the stimulatory effects were still observable on days 3 and 4 following injection. No effect was measurable on thymus cells.

Effect of Poly A:U on Cortison-resistant Spleen Cells

Inasmuch as poly A:U had not affected B-cells under our previously published experimental conditions, the above effect was hypothesized to be exerted on T-cells peripheralized to the spleen. To test whether poly A:U might be affecting selectively the peripheral T-cell population resistant to corticosteroids,[7] mice were given cortisone 2 days prior to the injection of poly A:U and/or SRBC. The results of 5 experiments are given in TABLE 4. In control mice receiving saline, poly A:U alone had a greater degree of mitogenic effect than the antigen (235% vs. 160% of the control value), whereas SRBC plus poly A:U gave an additive effect, approximately 330% of the control value. Mice that were given cortisone showed similar results, SRBC alone increasing [^{3}H]-thymidine uptake to 175% of the control, whereas poly A:U and poly A:U plus the antigen, showed 213% and 291% of the control results, respectively. Thus elimination of cortisone-sensitive lymphocytes did not reduce or modify the mitogenic effects of poly A:U, indicating an effect on other than the T_{II}-cells.

Effect on DNA Synthesis by Cortisone-resistant Spleen Cells In Vitro

To test whether or not the *in vivo* effects observed in the preceding experiments might be mediated through differences in homeostatic factors related to cortisone treatment, the following *in vitro* experiments were done. Spleen cells from mice treated with 2.5 mg cortisone 2 days prior to sacrifice were cultured;

TABLE 3

KINETICS OF MITOGENIC EFFECTS OF POLY A:U AND/OR SRBC ON MOUSE SPLEEN CELLS *in Vivo*

Mice * Injected with:		[^{3}H]Thymidine Incorporation Cpm (% cpm) †			
SRBC	Poly A:U	18 hours	2 days	3 days	4 days
−	−	4,460±670 (100±14)	1,800±210 (100±11)	2,600±20 (100±8)	1,380±400 (100±30)
−	+	3,420±1000 (77±21)	4,700±850 (260±47)	5,200±440 (200±16)	3,560±740 (260±52)
+	+	2,850±280 (63±6)	6,000±1150 (300±66)	5,250±800 (200±30)	3,350±400 (250±30)

* Mice were injected with 2×10^7 SRBC and/or 300 μg poly A:U intravenously 18 hours, 2, 3, or 4 days prior to the injection of [^{3}H]thymidine, which lasted for 2 hours.

† (Cpm exp. group/cpm control group) × 100 ± standard deviation.

TABLE 4

MITOGENIC EFFECTS OF POLY A:U AND/OR SRBC ON CORTISONE-RESISTANT SPLEEN CELLS *in Vivo* *

Mice † Injected with: First	Second	[³H]Thymidine Incorporation Cpm (% cpm) ‡ 86 §	87	88	89	93	Average % of cpm±SD
saline	saline	500 (100)	220 (100)	1265 (100)	545 (100)	250 (100)	100
saline	SRBC	680 (136)	250 (114)	2570 (200)	870 (160)	450 (180)	160±50
saline	poly A:U	935 (187)	600 (270)	3060 (240)	1160 (200)	740 (280)	235±41
saline	SRBC and poly A:U	1100 (220)	650 (300)	4450 (350)	2040 (374)	1050 (420)	330±76
cortisone	saline		310 (100)	1300 (100)	630 (100)	225 (100)	100
cortisone	SRBC		400 (129)	2850 (219)	1170 (185)	380 (169)	175±37
cortisone	poly A:U		860 (277)	2290 (176)	910 (144)	575 (256)	213±63
cortisone	SRBC and poly A:U		1020 (329)	4380 (337)	1400 (222)	625 (278)	291±53

* Spleens were removed 2 days after the 2nd injection, preceded by 2 hrs. [³H] thymidine pulse labeling.

† Mice were injected intraperitoneally with saline or 2.5 mg Hydrocortisone sodium succinate 2 days prior to second injection of 300μ poly A:U and/or 2×10^7 SRBC intravenously.

‡ (Cpm exp group/cpm control group) $\times$ 100 $\pm$ standard deviation.

§ Refers to experimental numbers.

10^6 SRBC and/or 4.5 μg poly A:U each was added to the culture medium containing 5×10^6 spleen cells, and the cells subsequently incubated for 48 hours, at which time 6 μci of [³H]thymidine/culture dish were added. Following an additional 18-hour culture period, cells were harvested and prepared for scintillation counting. TABLE 5 summarizes results from one of the typical experiments in this series. It may be noted that once again poly A:U increased the stimulatory effect somewhat. In cortisone-treated animals, an even greater level of DNA synthesis, was stimulated.

Effects of Poly A:U on Bone Marrow Cells

To determine whether the above described effects indeed were restricted to peripheralized T-cells in the spleen, bone marrow cells were incubated for 24 hours with 1 or 5 μg poly A:U in the presence or absence of SRBC. A 180% increase in [³H]thymidine incorporation was observed 18 hours after addition of 4 μci of the latter, irrespective of the dose level of poly A:U or

the presence or absence of the antigen. These figures were clearly greater than the values for SRBC alone, which produced only 135% of the control (TABLE 6).

DISCUSSION

The results obtained from the present investigation render additional support to preliminary data showing that mitogenic activity by poly A:U can be demonstrated in mouse spleen cells.[6, 8] Furthermore, it shows clearly that such stimulatory effects of poly A:U on [^{3}H]thymidine incorporation can be elicited in the absence of injected antigen (TABLE 3). Repeated experiments with cells obtained 2 days after the adjuvant and/or the antigen injection show that the combination of the two produces an additive effect (TABLE 4). The possibility that the mitogenic effect *in vivo* is induced within 6 hours has been raised by Cone and Johnson[6] who were able to suppress up to 90% of the immune response against SRBC with a single injection of vinblastine given 6 hours after the antigen plus poly A:U. Although similar enhancement effects were observed under *in vitro* conditions, the mitogenic effect of poly A:U under culture conditions was more consistent in the presence of antigen. This conclusion was supported also by Jaroslow and Ortiz-Ortiz[9] in their study of the effect of oligonucleotides on antibody synthesis *in vitro*.

Assuming that the surviving spleen cells following cortisone treatment are T-cells,[7] the observations made from both *in vivo* and *in vitro* experiments dealing with cortisone-treated animals (TABLES 4 and 5) indicate that cortisone-resistant T-cells may be responsible for the enhanced incorporation of [^{3}H]-thymidine. Indeed, the cells from cortisone-treated animals have shown a greater amount of [^{3}H]thymidine incorporation, particularly under *in vitro* conditions. Whether all of the B-cell population are killed by a single injection

TABLE 5

In Vitro INCORPORATION OF [^{3}H]THYMIDINE BY CORTISONE-RESISTANT SPLEEN CELLS

Substances Added into Culture			[^{3}H]Thymidine Incorporation	
Spleen Cells * from:	SRBC	Poly A:U	Cpm±SD	(% cpm±SD) †
normal	−	−	6,310±158	(100±3)
normal	+	−	10,240±700	(160±10)
normal	−	+	10,240±125	(160±3)
normal	+	+	13,510±4260	(210±7)
cortisone †	−	−	7,880±390	(100±5)
cortisone	+	−	13,100±146	(166±1)
cortisone	−	+	14,400±100	(183±0.7)
cortisone	+	+	23,000±2500	(292±11)

* Mice were injected intraperitoneally with saline or 2.5 mg hydrocortisone sodium succinate 2 days prior to sacrifice. 5×10^6 spleen cells in 0.5 ml MEM were incubated with 30 μl of 0.1% SRBC (1×10^6) and/or 0.5 μg poly A:U for 48 hours, followed by 6 μci of [^{3}H]thymidine added into the culture fluid and an additional 18 hours incubation.

† (Cpm exp. group/cpm control group) × 100 ± standard deviation.

TABLE 6

MITOGENIC EFFECTS OF POLY A:U ON MOUSE BONE MARROW CELLS *in Vitro*

Bone Marrow Cells * Incubated with:			[^{3}H]thymidine Incorporation	
SRBC	Poly A:U	LPS	Cpm±SD	(% cpm±SD) †
—	—	—	83,470±8,900	100±10
+	—	—	112,870±5,800	135±5
—	1 μg	—	152,870±12,900	180±8
—	5 μg	—	141,900±14,800	170±10
+	1 μg	—	152,300±22,400	180±15
+	5 μg	—	159,500±4,590	190±2
—	—	10^{-2} μg	197,000±41,700	235±21

* 5×10^6 bone marrow cells from normal mice were incubated with 30 μl of 0.1% SRBC (1×10^6) and/or 1–5 μg poly A:U or 10^{-2} μg LPS for 24 hrs., followed by 4 μci of [^{3}H]thymidine added into the culture fluid and an additional 18 hours incubation.

† (Cpm exp. group/cpm control group) × 100 ± SD.

of 2.5 mg cortisone at 2 days prior to poly A:U administration has not been determined, and thus this conclusion must be tentative. This is particularly pertinent in view of the fact that all our efforts at inducing mitogenicity in cells from the thymus and lymph nodes with poly A:U alone have produced negative results. However, T-cells in the circulation have different properties when compared to the T-cells present in the thymus.[10] Further fractionation and characterization of T-cell subpopulations are underway to resolve this issue.

The results of the *in vitro* experiments in which bone marrow cells were stimulated by poly A:U may not necessarily mean that poly A:U has the capacity to stimulate B-cells, in light of the recent data of Claman.[11] He has reported the presence of T-cells in mouse bone marrow as suggested by the positive response of the bone marrow cells towards specific T-cell mitogens such as phytohemagglutinin and concanavalin-A.

SUMMARY

Polyadenylic-polyuridylic acid complexes (poly A:U) at the 1–5 μg level, were mitogenic for spleen cells when given intravenously to normal Balb or cortisone-treated mice. Similarly, mitogenicity was evident when poly A:U was added to tissue culture fluids containing spleen cells from normal or cortisone-treated mice, or bone marrow cells from normal mice. Under these conditions, this adjuvant was not mitogenic for thymus cells or mesenteric lymph node cells, either *in vivo* or *in vitro*.

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DISCUSSION OF THE PAPER

DR. A. WHITE: Have you looked in the supernatants for cAMP? I ask this question because since the Bible has been mentioned I rise as a sinner. The fact that cAMP does not readily enter cells is well known. However, I should like to call attention to observations made in several laboratories that have indicated that, *in vitro*, certain lines of cells in culture export up to as much as three to five times as much cAMP as is synthesized during such an incubation period. I believe, therefore, that in any *in vitro* study one should be aware of this.

DR. A. G. JOHNSON: Although we are students of the Bible we have not yet gotten to Revelations and we hope that we can find out what the substance is. It is entirely possible, of course, that it might be a cAMP derivative. It does not negate the results.

DR. H. MILLER: I wish to ask if you have looked at the effects of poly A:U on the response to an antigen like polysaccharide or an antigen that is T-cell independent?

DR. A. G. JOHNSON: We did preliminary experiments along this line, and it showed no effect on thymus-independent antigens.

DR. H. MILLER: As second question, there is some suggestion that cellular resistance to infection by intracellular parasites is negated by T-cells acting on

macrophages. Is there any effect of poly A:U on the resistance of nude mice to some of these intracellular parasites?

DR. A. G. JOHNSON: We have not tested for that.

DR. G. MÖLLER: Do you believe that poly A:U works on T-cells in culture and is that the interpretation?

DR. A. G. JOHNSON: Yes, although we have not yet ruled out that we can use B-cells alone plus the T-cell factor and then determine whether the poly A:U acts on these cells.