Regulation of the Immune System By Synthetic Polynucleotides

IV. Amplification of Proliferation of Thymus-Influenced Lymphocytes [1]

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The role of thymus-influenced antigen reactive cells (ARC) in the immune response was studied with the aid of a potent adjuvant to both antibody formation and cell-mediated immunity, polyadenylic–polyuridylic acid complexes (poly A:U). The polynucleotide complex increased the anti-sheep erythrocyte rosette forming cell response of irradiated mice after thymus cells were exposed to poly A:U *in vivo* or *in vitro* prior to injection of bone marrow cells. This direct stimulatory action resulted in an enhancement of the rate of proliferation of ARC in response to antigen and a shortening of the interval between antigenic challenge and the initiation of division in immunocompetent cells. No stimulatory effect of poly A:U on bone marrow derived antibody forming precursor cells was observed.

It is suggested that ARC play an important role in the regulation of the length of the induction period, as well as the rate of increase of antibody forming cells in response to some antigenic stimuli.

INTRODUCTION

Complexes of polyadenylic acid and polyuridylic acid (poly A:U) have been found to enhance the immune response to sheep red blood cells (SRBC) in lethally irradiated mice reconstituted with a constant number of bone marrow cells (B cells) and graded numbers of thymus cells (T cells). The elevation in anti-SRBC rosette forming cells (RFC) was inversely proportional to the number of T cells injected. Conversely, poly A:U was not able to enhance the immune response of irradiated mice when injected with graded numbers of B cells and a constant number of T cells (1). Although the data suggested that the adjuvant action of poly A:U (2, 3) was mediated through an effect on T cells rather than through the B cells, the evidence was indirect. Direct evidence in support of a stimulatory effect of poly A:U on T cells was obtained in experiments described herein by exposing T cells alone to poly A:U either *in vivo* or *in vitro* prior to the addition

of B cells. The data revealed that one mode of action of this adjuvant was to increase the rate of proliferation of T cells and decrease the time at which cell division could be initiated by antigen.

MATERIALS AND METHODS

Animals. BALB/Aj mice originally obtained from Dr. William H. Murphy, Jr., Department of Microbiology, The University of Michigan (4) were inbred in our laboratory. Mice were either 4–5 weeks or 9–10 weeks old when used.

Antigen. Sheep red blood cells 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. In all instances mice received 4×10^8 SRBC intravenously.

Homoribopolynucleotides. Polyadenylic acid (poly A), potassium salt, (lot nos. 110748, 11–59–301) and polyuridylic acid (poly U), ammonium salt, (lot nos. 411454, 11–6–308) were purchased from Miles Laboratories, Elkhart, Indiana. Polymers were complexed in vitro to form poly A:U by mixing equal amounts of the polynucleotides before use as described previously (3).

Cell Preparations. Single suspensions of T cells, spleen cells or B cells were prepared as described previously (1). Thymus lobes or spleens were minced in cold PBS, pH 7.2, the cell suspension passed through a #80 gauge stainless steel mesh into PBS, washed 3× and resuspended in PBS, pH 7.2 to appropriate concentrations. Suspensions of bone marrow cells were prepared by flushing the marrow from femurs and tibias 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 cells washed 3× in cold PBS 7.2 and resuspended to an appropriate volume.

Thymectomy. Eighteen to 24 hr old mice were thymectomized according to the method of Sjodin *et al.* (5) as described previously (1). Thymic lobes were removed by aspiration. Prior to removal of spleens for rosette analysis the mice were examined with the aid of a dissecting microscope for the presence of thymic remnants. Mice with thymic remnants were excluded from the study.

Irradiation. Balb mice, 9–10 weeks old received 800R whole-body irradiation at a distance of 100 cm from a cobalt 60 source delivering 67R/min.

Drugs. Vinblastine sulfate (Eli Lilly and Company) was obtained from The University of Michigan Hospital pharmacy. The drug was dissolved in 0.85% (v/v) saline at a concentration of 1 mg/ml and stored without preservative at 4°C.

In vitro incubation of thymus cells and bone marrow cells. T or B cells were suspended to designated concentrations in Eagle's basal medium (BME) containing 2 mm D-glutamine and appropriate concentrations of SRBC ± poly A:U. Ten ml of the cell suspension were added to 50 ml siliconized screw-capped Erlenmeyer flasks. A gas mixture of 95 %air, 5% CO_2 was introduced into each flask and the suspensions were incubated at 37°C on a shaking water bath for 90 min. After incubation the cells were washed 4 times with 50–100 volumes of PBS, pH 7.2, resuspended in PBS, pH 7.2 and injected intravenously with antigen in 0.4 ml volumes.

Rosette Assay. Anti-SRBC antibody forming cells in the spleens of mice were

enumerated by the rosette assay as described previously (1). Spleens from each experimental group were pooled, dispersed, and washed in PBS pH 7.2. To 1 ml of the washed, monodisperse spleen cell suspension was added 0.1 ml of a 10% suspension of washed SRBC and this mixture was incubated for 1.5 hr at 37°C and overnight at 4°C. Rosette forming cells (RFC) were enumerated by counting the spleen cell–SRBC mixture in a hemocytometer at 200× magnification. Approximately 5,000 to 10,000 spleen cells were counted depending on individual experiments.

Criterion for determining positive and negative responding spleens. The criterion for designating whether or not an immune response was initiated in the spleens of irradiated mice receiving SRBC, excess B cells and limiting numbers of T cells was the appearance in the spleen of 10,000 or more RFC. This was based on the finding that negative control groups, composed of mice receiving only one of the two cell types plus antigen, had a mean of $4,200 \pm 1,300$ rfc/spleen, representing pooled data from a total of 25 mice.

RESULTS

Direct action of poly A:U on T cells. Both *in vivo* and *in vitro* experiments were done. In the former advantage was taken of the finding that poly A:U exerted its effect quickly and was rendered ineffective as an adjuvant in 10 min by mouse serum (3). Therefore, any direct effect on T cells alone ought to be revealed by injecting lethally irradiated mice with T cells, SRBC and poly A:U 1 day *before* injection of B cells and SRBC. Control groups of irradiated mice received B cells, SRBC and poly A:U 1 day before injection of T cells and SRBC. Within each group spleens were removed 8 days after the last injection of antigen, pooled and assayed for RFC. As may be seen in Table 1, poly A:U enhanced the RFC response approximately 3 fold when injected with 10^7 T cells and SRBC 1 day before B cells. In contrast little stimulation was achieved of the diminished immune response in irradiated mice receiving poly A:U and 10^7 B cells and antigen 1 day before T cells.

TABLE 1

In Vivo Amplification of T Cells By Poly A:U

Group [a]	Intravenous injection on day		RFC/spleen
	1	2	Day 10 [d]
1	10^7 T cells + SRBC [b]	10^7 B cells + SRBC	37,000
2	10^7 T cells + poly A:U [c] + SRBC	10^7 B cells + SRBC	99,000
3	10^7 B cells + SRBC	10^7 T cells + SRBC	10,000
4	10^7 B cells + poly A:U + SRBC	10^7 T cells + SRBC	15,000
5	10^8 T cells + SRBC	10^7 B cells + SRBC	91,000
6	SRBC	SRBC	0

[a] Mice received 800R whole-body irradiation on day 0.
[b] 4×10^8 SRBC/mouse.
[c] 600 μg poly A:U/mouse.
[d] Data represent pooled results of 2 separate experiments with 4–5 mice/group in each.

A second approach at demonstrating the stimulatory effect of poly A:U directly on thymus-influenced ARC involved incubating thymic lymphocytes *in vitro* with antigen and poly A:U for 90 min. After incubation, the T cells were washed 4 times and injected into irradiated mice in graded amounts with 10^7 B cells and SRBC. As may been seen in Fig. 1, mice which received SRBC and 5×10^5 thymic lymphocytes that had been incubated with poly A:U and SRBC, produced 6-fold more RFC than mice which received an equivalent number of T cells incubated with SRBC alone. As in a previous study (1) in which irradiated mice received T cells and poly A:U *in vivo*, the adjuvant effect of the polynucleotide complex decreased as the number of thymic lymphocytes injected was increased.

To determine whether poly A:U likewise might stimulate B cells *in vitro*, the latter were incubated with SRBC and poly A:U in an identical experiment. As may be seen in Table 2, a much lesser stimulatory effect of poly A:U was observed when irradiated mice received 2×10^6 or 1×10^7 B cells that had been incubated with poly A:U and SRBC. In contrast the amplifying action of the polynucleotide complex on T cells was evidenced once again by the control group of irradiated mice which received 10^6 thymic lymphocytes that had been incubated with poly A:U and SRBC. Ten-fold more RFC were seen in these mice.

Nature of the stimulatory effect of poly A:U on thymus-influenced ARC. It was not apparent whether poly A:U expanded the initial number of ARC or whether the homoribopolymer complex amplified the activity of each existing ARC which reached the spleen in the above experiments. To differentiate between these two possibilities, the response within each individual mouse spleen was studied. Since
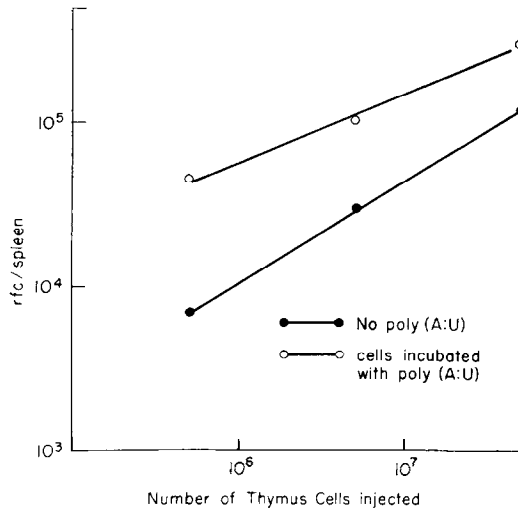

FIG. 1. Restoration of immunocompetence in irradiated mice injected with bone marrow cells and graded numbers of thymus cells incubated *in vitro* with SRBC ± poly A:U. Mice receive 800R whole-body irradiation and 1 day later 1×10^7 bone marrow cells, 4×10^8 SRBC and graded numbers of thymus cells. 5×10^7/ml of the latter were incubated *in vitro* with 5×10^7/ml SRBC ± poly A:U at 150 µg/ml. Spleens were removed on day 9, pooled and assayed for RFC. Each point represents the data obtained from a pool of 5–6 spleens.

the proportion of irradiated mice exhibiting spleens with a positive immune response after receiving excess B cells, antigen and limiting numbers of T cells depends on the number of injected T cells (6), T lymphocytes were incubated with antigen and poly A:U, and 10^6 or 3×10^7 cells were injected with SRBC and 10^7 B cells into irradiated recipients. Splenic RFC were assayed in individual spleens on day 9. Fifty percent of the mice receiving 10^6 T lymphocytes incubated with antigen alone yielded positively responding spleens (as defined in Materials and Methods), while 70% of the mice receiving T cells that had been incubated with SRBC and poly A:U exhibited positive spleens. Thus, poly A:U did not appear to increase significantly the number of ARC reaching the spleen. However, although the number of mice responding was not increased markedly by poly A:U the positively responding spleens from mice receiving 10^6 T lymphocytes that had been incubated with poly A:U contained 6-fold more RFC than positive spleens of mice receiving an equivalent number of T cells incubated with antigen alone. These

TABLE 2

RESTORATION OF IMMUNOCOMPETENCE IN LETHALLY IRRADIATED MICE BY THYMUS CELLS
INCUBATED IN VITRO WITH SRBC AND POLY A:U

Group [a]	Thymus cells injected	Bone marrow cells injected	RFC/spleen
1	4×10^7	1×10^7 (incubated with SRBC)	150,000
2	4×10^7	1×10^7 (incubated with SRBC and poly A:U)	220,000
3	4×10^7	2×10^6 (incubated with SRBC)	32,000
4	4×10^7	2×10^6 (incubated with SRBC and poly A:U)	25,000
5	1×10^6 (incubated with SRBC)	1×10^7	5,500
6	1×10^6 (incubated with SRBC and poly A:U)	1×10^7	60,000
7	1×10^6	1×10^7 (incubated with SRBC)	8,200
8	1×10^6	1×10^7 (incubated with SRBC and poly A:U)	6,900
9	---	—	6,600

[a] Mice received 800R whole-body irradiation and 24 hr later 4×10^8 SRBC + thymus and bone marrow cells IV. Spleens were assayed on day 9 for RFC. Each group represents data obtained from a pool of 5-6 spleens/group. Culture conditions: thymus cells—5×10^7 SRBC/ml $\pm$ 150 μgms poly A:U/ml; bone marrow cells—1×10^7/ml + 10^7 SRBC/ml $\pm$ 150 μgms poly A:U/ml.

results suggested that poly A:U amplified the activity of those ARC which reached the spleen.

To examine further the nature of this amplifying effect, a double transfer experiment similar to that used by Mitchell and Miller (7) was employed. In this type of experiment it was shown that T cells injected into an irradiated mouse (primary host) will seed the spleen and proliferate in response to antigenic challenge (8). The activity of these cells may then be assessed by transferring spleen cells from the primary host along with antigen and fresh B cells to a second irradiated mouse (secondary host). Since the number of RFC produced in irradiated mice receiving SRBC, an excess number of B cells and graded numbers of T cells, was proportional to the number of thymus cells injected (1), any increase in RFC in the spleens of secondary hosts, receiving primary host spleen cells removed at various times after injection of T cells and antigen, should be a reflection of the increase in thymus influenced ARC in the spleens of primary hosts. Accordingly, such a double transfer experiment was used to determine what effect poly A:U had on the proliferative rate of ARC responding to antigenic challenge.

Lethally irradiated mice (primary hosts) received 2×10^7 T lymphocytes, SRBC and poly A:U . Their spleens were removed 24, 48 and 72 hr after injection, the cells dispersed, pooled and 10^7 spleen cells were injected with 10^7 B cells and SRBC into a second group of lethally irradiated mice (secondary hosts). Eight days after the secondary hosts received primary host cells, B cells and antigen, the spleens of secondary host mice were removed and assayed individually for RFC. No immune response was detected in secondary hosts injected with SRBC, B cells and spleen cells obtained from primary hosts injected with SRBC alone, indicating that no ARC were present in the irradiated primary host. As may be seen in Fig. 2 injection of SRBC, B cells and spleen cells obtained from primary hosts 24 hr after injection of SRBC and T cells, enabled 20% of the secondary hosts to respond to antigenic challenge. This number of ARC in the spleens of primary hosts receiving T cells and SRBC increased between 24 and 48 hr as evidenced by the finding that 80% of the mice yielded positive spleens when receiving primary host spleen cells removed 48 hr after injection of SRBC and T cells. That the T cells were proliferating in response to antigenic challenge was indicated by the observation that there was little increase in the proportion of secondary hosts receiving spleen cells removed from primary hosts 24 or 48 hr after injection of T cells without antigen. Sixty percent of the secondary hosts receiving SRBC, B cells and spleen cells obtained from primary hosts 24 hr after injection of SRBC, T cells and poly A:U responded to antigenic challenge, indicating a greater number (60% vs 20%) of ARC in the spleens of primary hosts receiving poly A:U. This number of ARC in primary hosts receiving poly A:U also increased between 24 and 48 hr after injection such that spleen cells removed from poly A:U-treated primary hosts 48 hr after injection enabled 100% of the secondary hosts to respond to antigenic challenge. That the polynucleotide complex had amplified the activity of ARC in primary hosts was also indicated by the observation that spleen cells removed from poly A:U treated primary hosts enabled the production of 2 to 6 fold more RFC/positive spleen than spleen cells obtained from primary hosts receiving SRBC and T cells alone. Furthermore, the number of RFC/positive spleen in secondary
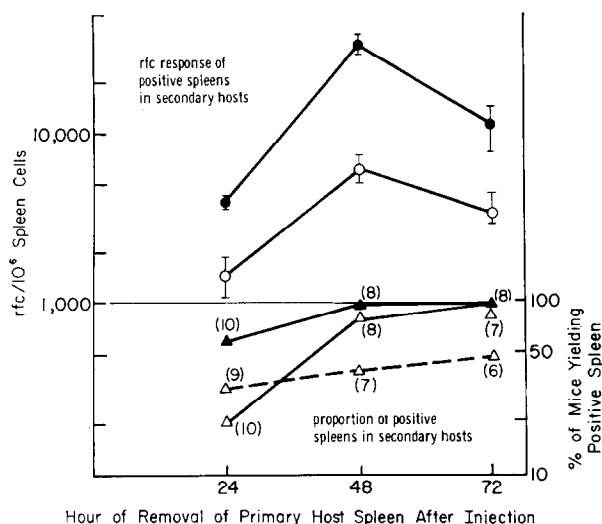
Fig. 2. RFC formation in irradiated secondary host mice injected with SRBC, bone marrow cells and spleen cells removed from irradiated primary host mice. Irradiated mice (800R) received 4×10^8 SRBC, 1×10^7 bone marrow cells and 1×10^7 spleen cells obtained from irradiated mice 24, 48, or 72 hr after IV injection of 2×10^7 thymus cells, 4×10^8 SRBC $\pm$ 600 μg poly A:U. Spleens of secondary hosts were removed 8 days after injection of antigen and assayed individually for RFC. Each point represents the pooled results of 2 separate experiments. Vertical bars denote 1 SE of arithmetic mean. ●———● 2° host receiving SRBC, Bone Marrow Cells and Spleen Cells from ▲———▲ 1° host injected with SRBC, Poly A:U and Thymus Cells. ○———○ 2° host receiving SRBC, Bone Marrow Cells and Spleen Cells from △———△ 1° host injected with SRBC and Thymus Cells. △---△ 2° receiving SRBC, Bone Marrow Cells and Spleen Cells from 1° host injected with Thymus Cells.

hosts receiving spleen cells removed from primary hosts 48 hr after injection of antigen and T cells was 4-fold higher than the number of RFC induced by spleen cells removed 24 hr after injection. Primary host spleen cells removed 48 hr after injection of SRBC, T cells and poly A:U induced 10 fold more RFC than spleen cells removed from poly A:U-treated primary hosts removed at 24 hr. If the increase in RFC is an accurate reflection of the increase in ARC, the number of ARC in poly A:U treated primary hosts doubled in approximately 7 hr, while ARC in primary hosts injected with T cells and antigen alone doubled in 12 hr.

Thus, the results suggested that poly A:U increased the rate of proliferation of ARC in response to antigenic challenge. In addition, the increased number of ARC in the spleens of irradiated mice within 24 hr after injection of T cells, SRBC and poly A:U indicated that antigen-stimulated division of ARC may have begun earlier in poly A:U -treated animals.

Amplification of ARC by poly A:U, as demonstrated in the irradiated–reconstituted mouse model, might also explain the restoration by poly A:U of immunocompetence in neonatally thymectomized (NTx) mice (1). To test this hypothesis, the rate of increase in antibody producing cells was determined as well as the time at which the immune response of poly A:U-treated NTx mice became susceptible to

the inhibitory effects of vinblastine (VLB). Thus, five week old NTx mice received SRBC ± poly A:U or T lymphocytes. Spleens were removed at various times after injection of antigen and assayed for RFC. As may be seen in Fig. 3, the number of splenic RFC in NTx mice receiving antigen alone doubled in 24 hr and there was little further increase in RFC after day 3. When NTx mice received SRBC and poly A:U or thymic lymphocytes, splenic RFC increased exponentially to day 4 with a doubling time of 18 hr. The number of RFC in NTx mice receiving poly A:U and SRBC increased further between days 4 and 6 while the increase was not extended in unoperated mice or NTx mice receiving antigen and thymic lymphocytes. Thus, the rate of increase of antibody forming cells in NTx mice was enhanced by poly A:U.

The possibility that the homoribopolymer complex stimulated earlier initiation of division of immunocompetent cells was examined with the use of a mitotic inhibitor, vinblastine. Since dividing cells are inhibited by VLB (9, 10), this drug has been used to determine when immunocompetent cells began to divide after injection of antigen (8, 11). Prior to determining the effect of poly A:U in VLB-treated NTx mice, the effect of the polynucleotide complex was studied in VLB-treated, unoperated animals. Five week old, unoperated mice received intravenously, SRBC and poly A:U and 6, 12 or 24 hr later 0.05 mg VLB intraperitoneally. Spleens were removed 3 days after injection of antigen and assayed for RFC. As may be seen in Fig. 4, VLB suppressed the immune response of mice receiving antigen alone when injected 12 hr or more after injection of antigen. In contrast, 93% suppression of the immune response was achieved when VLB was injected 6 hr
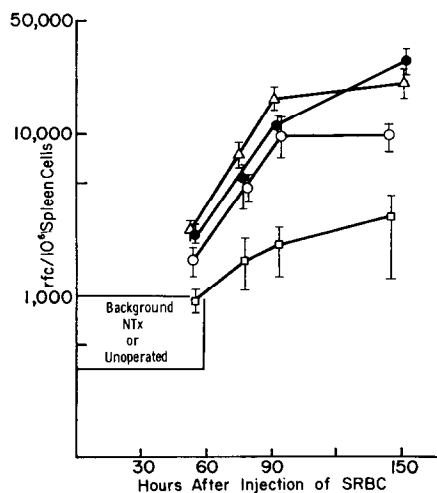

Fig. 3. Kinetics of splenic RFC response to SRBC in NTx and unoperated mice. Mice were injected intravenously with 4 × 10⁸ SRBC either alone or with 5 × 10⁷ thymic lymphocytes or 600 μg poly A:U. Spleens were removed at designated intervals after injection of antigen and assayed for RFC. Each point represents the arithmetic mean of data obtained from 4–5 mice/group. Vertical bar represents 1 SE of arithmetic mean. □——□ NTx mice injected with SRBC. ○——○ NTx mice injected with SRBC + thymus cells. ●——● NTx mice injected with SRBC + poly A:U. △——△ Unoperated mice injected with SRBC.
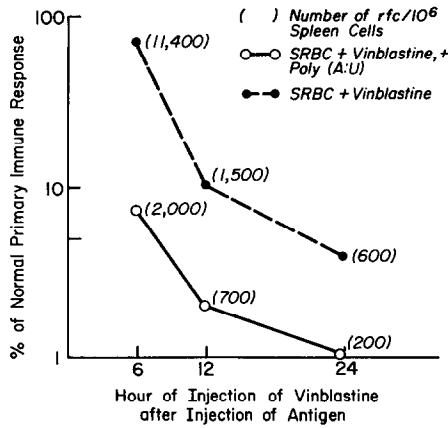
FIG. 4. Early effect of vinblastine on the immune response of mice injected with SRBC and poly A:U. Mice were injected intravenously with 4×10^8 SRBC + 600 μg poly A:U and intraperitoneally with 0.05 mg vinblastine 6, 12, or 24 hr later. Spleens were removed on day 3, pooled and assayed for RFC. Each point represents data obtained from 3–4 mice/group.

after injection of SRBC and poly A:U. Similarly, as may be seen in Table 3, VLB suppressed the immune response of NTx mice when injected 12 hr or more after injection of SRBC. When NTx mice received SRBC and poly A:U, VLB inhibited the immune response when given with, or 6 hr or more after, injection of antigen. These results suggested that antigen induced proliferation of immunocompetent cells began at least 6 hr earlier in poly A:U treated NTx or unoperated animals.

DISCUSSION

A direct, stimulatory effect of poly A:U on thymus-influenced ARC was demonstrated by the finding that incubation of thymus cells with SRBC and poly A:U

TABLE 3

EFFECT OF VINBLASTINE ON POLY A:U-INDUCED RESTORATION
OF IMMUNOCOMPETENCE OF NTx MICE

Inoculum [a]	Poly A:U-treated NTx mice		Untreated NTx mice	
	RFC/10^6 spleen cells	% reduction	RFC/10^6 spleen cells	% reduction
SRBC	4,500	—	1,400	—
SRBC + VLB				
0 hr	680	85	1,500	0
6 hr	650	86	1,500	0
12 hr	440	90	430	65
24 hr	620	88	260	89

[a] NTx mice received 4×10^8 SRBC $\pm$ 600 μg poly A:U IV and 0.05 mg vinblastine IP 0, 6, 12 or 24 hr after injection of antigen. Spleens were removed on day 3 and assayed for RFC. Each group represents the data obtained from a pool of 3–5 spleens. VLB = vinblastine sulfate.

in vivo and *in vivo* prior to injection of B cells enhanced the anti-SRBC response of irradiated mice. In contrast, the inability of the homoribopolymer complex to stimulate B cells *in vivo* or *in vitro* indicated that poly A :U does not exert directly, an adjuvant effect on AFPC of bone marrow origin. The observation that no adjuvant effect was achieved when irradiated mice received 10^6 thymic lymphocytes, SRBC and 10^7 B cells with only the latter exposed to poly A :U suggested further that the inability to demonstrate an adjuvant effect on B cells was not due to masking of the effect by injection of large numbers of T cells.

The magnitude of the increase in frequency of positively responding mice injected with antigen, excess B cells and limiting numbers of poly A :U-treated T cells was too small to account for the increased number of antibody forming cells in the spleens of responding mice. Thus, the major effect of poly A :U would appear to be to amplify the activity of those ARC which have reached the spleen of the irradiated animal. The finding that injection of poly A :U with SRBC did not increase significantly the number of anti-SRBC hemolytic foci/spleen in normal animals, but rather increased the number of plaque forming cells/foci (12) is in agreement with this hypothesis. The amplification of ARC by poly A :U might be brought about in several ways: (a) increased rate of proliferation of ARC, resulting in increased numbers and rate of activation of AFPC, (b) increased numbers of divisions of ARC, (c) increased efficiency of individual ARC such that an individual ARC might interact with a larger number of AFPC.

The data obtained from double transfer experiments suggested that poly A :U enhanced the rate of increase of ARC in response to antigenic challenge. The theoretical doubling time of ARC calculated from data obtained in double transfer experiments is in agreement with that obtained by others (8). Treatment of irradiated primary hosts with poly A :U resulted in a shortening of the doubling time of ARC by 5 hr. Similarly, the doubling time of RFC in NTx mice treated with poly A :U was decreased by 6 hr. The results suggested that at least one of the factors affecting the rate of proliferation of antibody forming cells may be the proliferative rate of ARC. In support of this concept, the rate of recruitment of antibody forming cells was found to proceed exponentially (11). Thus, the increased proliferative rate of RFC in poly A :U-treated NTx mice might be a reflection of an increase in the rate of proliferation of ARC. The susceptibility of mice receiving poly A :U and antigen to the immunosuppressive effects of VLB within 6 hr after injection of antigen suggested that the homoribopolymer complex also induced an earlier proliferation of immunocompetent cells in response to antigenic challenge. Since the antigen-induced expansion of ARC may be inhibited by VLB (8), it is probable that poly A :U stimulated even earlier divisions of ARC. These data offer a likely explanation for the early appearance of antibody in the blood stream of poly A :U treated animals (3).

The nature of the interactions between ARC and antigen and/or AFPC is unknown. The ability of poly A :U to amplify the activity of ARC suggests that the homoribopolymer complex might be a useful tool in defining the precise role these cells play during the immune response. In addition, the ability of the homoribopolymer complex "to restore" immunocompetence to animals rendered deficient in thymus-influenced cells holds promise as a means of therapy for individuals suffer-

ing from immunologic deficiency syndromes in which the thymus is not absent but is poorly developed (13).

ACKNOWLEDGMENT

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