REGULATION OF THE IMMUNE SYSTEM BY SYNTHETIC POLYNUCLEOTIDES

III. ACTION ON ANTIGEN-REACTIVE CELLS OF THYMIC ORIGIN*

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Interaction of several cells has been shown to be required in the immune response of mice to sheep red blood cells (SRBC) (1, 2). The bone marrow-derived lymphocyte apparently functions as the antibody-forming precursor cell (AFPC) after contact in an unknown fashion with a thymic-influenced lymphocyte (2–5). Thus, when antibody synthesis in neonatally thymectomized (NTx) mice was restored by injection of donor, viable, thymic lymphocytes, 19S hemolysin-producing cells in the spleen of the recipient were shown to be derived from the host and of bone marrow origin (2). However, while thymic lymphocytes may not differentiate to become antibody-forming cells in this system, they nevertheless respond to antigenic stimulation by proliferation (6, 7), and have been termed antigen-reactive cells (ARC).

Evidence that the macrophage may be one of the cell types stimulated by the potent adjuvant action of homoribopolynucleotide complexes (8, 9) was recorded in the preceding manuscript (10). The effect of polyadenylie-polyuridylic acid complexes (poly A:U) on thymic-influenced and bone marrow-derived cells in the model system of Miller and Mitchell (2) are presented herein, and the experiments described suggest that an additional mode of action of

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1 Abbreviations used in this paper: AFPC, antibody-forming precursor cell; ARC, antigen-reactive cell; ATS, anti-mouse thymocyte serum; NTx, neonatally thymectomized; PBS, phosphate-buffered saline; poly A, polyadenylic acid; poly A:U, polyadenylic-polyuridylic acid complex; poly U, polyuridylic acid; SRBC, sheep red blood cells.

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poly A:U in enhancing antibody synthesis is to expand a small population of thymic-influenced cells. No effect on bone marrow cells was apparent.

Materials and Methods

Animals.—BALB/Aj mice originally derived from those of Dr. William Murphy, Department of Microbiology, The University of Michigan (11), were inbred in our laboratory. C57Bl/6j mice were obtained from Jackson Laboratories, Bar Harbor, Maine. The mice were fed Purina mouse chow and water ad libitum. New Zealand white rabbits approximately 1-2 kg were obtained from a local supplier.

Antigen.—SRBC were obtained from the Colorado Serum Company, Denver, Colo. The cells were stored at 4°C in modified Alsever’s solution, washed three times, and resuspended in phosphate-buffered saline (PBS), pH 7.2, for injection. In all instances mice received 4 × 10⁸ SRBC intravenously.

Homoribopolyribonucleotides.—Polyadenylic acid (poly A) potassium salt (lots 110748, 11-S7-301) and polyuridylic acid (poly U) ammonium salt (lots 411754, 11-6-308) were purchased from Miles Laboratories, Elkhart, Ind. Each polymer was stored at −20°C at a concentration of 15 mg/ml in PBS, pH 7.2. Polymers were complexed in vitro to form poly A:U by mixing equal amounts of the polynucleotides before use.

Heterologous Anti-Thymocyte Serum.—Anti-mouse thymocyte serum (ATS) was prepared by the method of Gray et al. (12). Single cell suspensions of thymic lymphocytes from 5-wk old female BALB/Aj mice were washed three times and resuspended in PBS, pH 7.2. The washed suspension was mixed with an equal volume of Freund’s complete adjuvant (Difco Laboratories, Detroit, Mich.) and injected into the footpads of NZW rabbits such that each rabbit received 1 × 10⁶ thymic lymphocytes. 4 wk later the rabbits received intravenous injections of thymic lymphocytes in PBS for 3 consecutive days for a total of 3 × 10⁸ cells. The rabbits were bled 7 and 10 days after the late injection. Each serum was absorbed with SRBC and BALB/Aj mouse RBC and stored at −20°C. The antiserum was found to agglutin-
nate thymic lymphocytes to a dilution of 1:512. 0.4 ml or more antiserum injected intra-
peritoneally 2-3 days before an intravenous injection of SRBC resulted in maximum (90-95%)
suppression of the immune response to SRBC as measured by the rosette technique. In all
cases the serum was inactivated by heating at 56°C for 30 min before use.

Cell Preparations.—Single cell suspensions of thymic lymphocytes used to prepare ATS or to
restore thymectomized or irradiated mice were obtained by mincing the thymic lobes of 4-5-
wk old BALB/Aj mice in cold PBS, 7.2. The cell suspension was passed through a No. 80
gauge stainless steel mesh into PBS, washed three times, and resuspended in PBS, pH 7.2, to
appropriate concentrations. Suspensions of bone marrow cells were prepared from 9-10-wk
old Balb/Aj mice. The marrow was flushed from femurs and tibias by means of a syringe and a
26 gauge needle containing cold Eagle's basal medium. The extruded plugs were dispersed
by aspiration with a 21 gauge needle and the cells were washed three times in cold PBS,
7.2, and resuspended to an appropriate volume.

Thymectomy.—18-24-hr old mice were thymectomized according to the method of Sjodin
et al. (13). Mice were anesthetized with ether and a longitudinal incision was made into the
mediastinal cavity to the level of the fourth rib. The thymic lobes were removed by aspiration
and the wound was closed with a single silk suture. Before 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.

Transplantation.—BALB/Aj mice were the recipients of skin from 5-6-wk old female
CS7BL/6j mice. Recipient mice were anesthetized with pentobarbital anesthesia and the skin
was removed from their backs in whole thickness in the shape of a rectangle approximately
2 × 2 cm. A skin fragment of a slightly larger size was removed from the abdomen of the
donor and sutured in place using 5-0 surgical thread. The mice were housed separately and
observed each day for signs of rejection.

Irradiation.—BALB/Aj mice, 9-10-wk old were exposed to 800 R whole-body irradiation
at a distance of 100 cm from a 60Co source delivering 67 R/min.

Rosette Assay.—6 days after injection of SRBC, the immune response to SRBC was meas-
ured by the rosette assay (14, 15). Spleens from each experimental group were pooled, dis-
persed, and washed in PBS, pH 7.2. To 1 ml of the washed, monodisperse spleen cell sus-
pension was added 0.1 ml of a 10% suspension of washed SRBC; this mixture was incubated for
1.5 hr at 37°C and overnight at 4°C. SRBC adhere to cells producing anti-SRBC antibody to
form a “rosette.” Rosette-forming cells (RFC) were enumerated by counting the spleen cell–
SRBC mixture in a hemocytometer at 200 × magnification. Approximately 5,000–10,000
spleen cells were counted, depending on individual experiments.

RESULTS

Restoration of the Immune Response to SRBC in NTx Mice by Thymic Lym-
phocytes or Poly A : U.—The ability of thymic lymphocytes to restore in our
system an immune response impaired by neonatal thymectomy is shown in
Fig. 1. It may be seen that NTx reduced by 90% the numbers of RFC occurring
in response to SRBC, while 10⁶ thymic lymphocytes restored the response of
NTx mice back to 54% of normalcy.

In testing whether poly A : U exerted its adjuvant action on the thymic
lymphocyte in this system, the effect of poly A : U alone, without lymphocytes
in NTx mice, also was determined. Surprisingly, NTx mice injected intra-
venously with SRBC plus poly A : U at a concentration of 1200 µg (600 µg of
each polymer) were restored on day 6 to full immunocompetence with respect
to RFC, in the absence of injected thymic lymphocytes (Fig. 2). As the quantity of poly A:U was lessened, the number of RFC produced decreased as a straight-line function.

FIG. 2. Restoration of immune response in neonatally thymectomized mice by poly A:U. Mice received $4 \times 10^8$ SRBC ± poly A:U intravenously. Spleens were removed 6 days later and pooled for RFC assay. Each point represents the arithmetic mean of three separate experiments with four to five mice per group in each experiment.

| Mice injected with | Mean survival time (days) | Unoperated | Tx |
|--------------------|---------------------------|------------|----|
| —                  | 13.5 (±0.8) *             | 6          | 24.0 (±3.0) | 7 |
| Poly A:U           | 12.0 (±1.3)               | 6          | 13.0 (±3.2) | 8 |

* () indicates 95% confidence interval.

BALB/Aj recipients received whole-thickness skin grafts from C57BL/6j donors. Mice receiving poly A:U were injected with 600 µg intraperitoneally 12, 24, and 72 hr after grafting.

Since neonatal thymectomy depresses cellular immunity as well as circulating antibody to SRBC, the experimental procedure was extended to determine whether NTx mice treated with poly A:U alone would regain the capacity to reject skin homografts. Accordingly, NTx and unoperated BALB/Aj mice were grafted with skin from C57BL/6j donors. Groups of NTx and unoperated recipients received 600 µg of poly A:U intraperitoneally 12, 24, and 72 hr after
As can be seen in Table I, skin grafted on mice with an intact thymus had a mean survival time of 13.5 days, while the mean survival time of grafts on NTx mice was extended to 24 days. However, NTx mice which received poly A:U were able to reject grafts at the same rate as unoperated mice. Thus, cellular as well as humoral immunity appeared to be restored by poly A:U in NTx mice, corroborating earlier studies on the effect of poly A:U on cell-mediated immune reactions (16).

**Fig. 3. Restoration of immune response in ATS-treated mice by poly A:U.** Mice received $4 \times 10^8$ SRBC ± poly A:U intravenously 3 days after intraperitoneal injection of 0.4 ml ATS. Spleens were assayed for RFC 6 days after injection of antigen. Each point represents the arithmetic mean of three separate experiments with three to four mice per group in each experiment.

To confirm the competence of poly A:U in overcoming inhibiting effects on lymphocytes, a different approach was utilized where injection of ATS was substituted for neonatal thymectomy. Accordingly, varying doses of poly A:U along with SRBC were injected into normal mice 3 days after they received intraperitoneally 0.4 ml of ATS. Splenic RFC were assayed 6 days after the injection of antigen. As can be seen in Fig. 3, untreated mice produced 42,000 RFC/10⁶ spleen cells while mice pretreated with ATS produced only 5% of this number. 1200 µg of poly A:U increased the response of ATS-treated mice 10-fold. As was the case with poly A:U administered to NTx mice, when the quantity of poly A:U was lessened, the number of RFC produced decreased.

From the preceding data it was evident that poly A:U injection could readily replace the need for thymic lymphocytes in animals depleted of such cells. It was not apparent, however, whether the restorative effects of poly A:U con-
stituted an actual replacement of a polynucleotide-like hormone from thymic lymphocytes or whether its effects were dependent on the amplification of a small number of residual thymic cells which might have seeded or influenced the peripheral lymphoid tissue before thymectomy.

In testing this question, it was reasoned that if both thymectomy and ATS treatment were combined, the number of any residual cells bearing thymic influence would be decreased further than with either treatment alone. If poly A:U action was based on amplifying these cells, its effect should have been reduced if the number of cells to be amplified were reduced. If poly A:U were simply replacing the need for hormone action on a nonthymic cell, then decreasing the residual cells remaining after thymectomy should have no effect on poly A:U enhancement. The results agreed with the amplification hypothesis

| Products injected | RFC/10⁶ spleen cells |
|-------------------|---------------------|
|                   | Unoperated | Thymectomized only | ATS only | Thymectomized + ATS |
| SRBC              | 23,000      | 1600               | 2000     | 615                 |
| SRBC + poly A:U   | N.D.        | 11,700             | 9700     | 2000                |

Mice received 4 × 10⁸ SRBC ± 600 µg poly A:U intravenously 3 days after intraperitoneal injection of 0.4 ml ATS. Values represent arithmetic mean of three separate experiments with four to five mice per group in each experiment.

N. D. = not done.
derived and bone marrow-derived cells to produce an immune response to SRBC (1), this model system was employed to distinguish any possible effects of poly A:U on thymic ARC and/or AFPC of bone marrow origin. Thus, unoperated mice which received 800 R whole-body irradiation 24 hr previously were injected with either $4 \times 10^9$ SRBC, SRBC and thymic lymphocytes, SRBC and bone marrow cells, or SRBC and a combination of the two cell types. Spleens were assayed for RFC 9 days after the injection of antigen.

No increase in RFC was found in the spleens of irradiated mice which received SRBC only or SRBC plus bone marrow cells, as expected. Mice which received SRBC and a combination of thymic lymphocytes and bone marrow cells had $1.8 \times 10^6$ RFC/spleen which was 63 times as many RFC as mice receiving SRBC and thymic cells only, $2.9 \times 10^6$ RFC. Thus, the need for both bone marrow cells and thymic-influenced lymphocytes in the restoration of immunocompetency under these conditions was demonstrated.

With this established, the possibility that poly A:U could amplify the function of small numbers of ARC was studied by injecting lethally irradiated mice with poly A:U and graded numbers of thymic lymphocytes plus a constant number in excess of bone marrow cells and antigen. Using minimal replacement levels of thymus cells ($4 \times 10^4$-$5 \times 10^6$), a pronounced effect of poly A:U on thymic cells was seen, as is shown in Fig. 4. Thus, the addition of poly A:U to

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just 40,000 thymic lymphocytes enabled the expression of an immune response in irradiated mice receiving bone marrow cells and SRBC. This low number of thymocytes without poly A:U was nonfunctional. The immunoenhancing effect of poly A:U decreased as the number of thymic lymphocytes injected was increased, suggesting that with higher numbers of thymus cells, the number of bone marrow cells became a limiting factor in the expression of an immune response. Thus, when ARC were present in excess of AFPC, the maximum number of thymus–marrow cell interactions probably occurred, and further increases in the number of ARC would not elevate the number of RFC produced.

To test for any effect of poly A:U on bone marrow cells, irradiated mice were injected in like experiments with graded numbers of bone marrow cells, SRBC, poly A:U, and a constant number, $10^6$, of thymic lymphocytes. Spleens were removed on day 9 and assayed for RFC. As can be seen in Fig. 5, the num-

![Graph](image-url)

**Fig. 5.** Effect of poly A:U on the immune response of irradiated mice receiving graded numbers of marrow cells and a constant number of thymus cells. Mice received 800 R whole-body irradiation and 24 hr later received intravenously $10^8$ thymic lymphocytes, $4 \times 10^8$ SRBC, graded numbers of bone marrow cells, and 600 µg poly A:U. Spleens from each group were removed 9 days after injection of cells and antigen, pooled, and assayed for RFC. The average number of RFC/spleen was determined by computing the total number of RFC/number of mice in each group. Each point represents the results obtained with five to six mice per group.
The number of RFC per spleen increased from $5 \times 10^3$ to $1.2 \times 10^5$ as the number of bone marrow cells injected was increased from $2 \times 10^6$ to $4 \times 10^7$. However, when poly A:U was injected with $2 \times 10^6$ or $10^7$ bone marrow cells, no adjuvant effect was observed. Only when the number of bone marrow cells was increased to $4 \times 10^7$, such that the thymocytes became limiting, did a rise in RFC become apparent with poly A:U treatment. This probably reflected action on the thymocyte and not the bone marrow cell.

**DISCUSSION**

The ability of poly A:U to restore the RFC response in NTx- or ATS-treated mice supports the hypothesis that this adjuvant exerts its action on the thymic-influenced ARC, in addition to the effect on the macrophage recorded in the preceding manuscript (10). That thymectomy or ATS treatment reduced the number of thymic-influenced ARC necessary for the full expression of immunocompetence is documented by the ability of thymic lymphocytes to restore RFC as well as the PFC response of NTx- or ATS-treated mice (2, 17, 18). As RFC have been shown to be actively synthesizing antibody (19, 20) and antibody-forming cells are of bone marrow rather than thymic origin (3-5), it is unlikely that thymic lymphocytes were functioning as RFC precursors, but rather as ARC. That thymic lymphocytes in this system do not end up as RFC is also suggested by the fact that irradiated mice which received a combination of thymic lymphocytes and bone marrow cells produced 63-fold more RFC than irradiated mice receiving thymic lymphocytes and SRBC only. The observation (8) that poly A:U shortened the time required for the attainment of the peak number of PFC responding to SRBC might suggest that the polynucleotides stimulated the proliferation of AFPC after such cells interacted with ARC. This is probably not the case insasmuch as poly A:U injected 1 day after antigen in normal mice did not exert any adjuvant effect. 2

Experiments by Shearer and Cudkowicz indicate that one of the first steps in the initiation of the immune response to SRBC is an antigen-dependent proliferation of thymic-derived ARC (7). Accordingly, poly A:U might influence the rate of activation and/or proliferation of ARC, thus amplifying the small number of ARC which were present in the peripheral lymphoid tissue before, and persisting after thymectomy, as attested by the further reduction in RFC in NTx mice by ATS treatment. Such a possibility is supported by the observation that poly A:U allowed the detection of an immune response in irradiated mice receiving bone marrow cells, SRBC, and as few as 40,000 thymic lymphocytes. The decreased adjuvant action of poly A:U as the bone marrow cell population became limiting, further emphasizes that thymic lymphocytes, rather than the bone marrow cells, are stimulated by the homoribopolymers. In addition, experiments in progress in which thymic lymphocytes were incubated with poly A:U in vitro before injection into thymectomized
mice indicate that poly A:U exerts its stimulatory effect directly on thymic lymphocyte membranes when increasing immunocompetence in NTx mice.

The restoration of immunocompetence in NTx mice by the implantation of thymus organs in diffusion chambers (21, 22) or the injection of cell-free extracts of thymic tissue (23, 24) has led to the suggestion that the thymus may secrete a competence-inducing humoral factor. This raises the question whether the polynucleotides act like a thymic hormone. However, the lessening of the adjuvant action of poly A:U as the number of residual thymocytes in NTx mice was decreased further by ATS treatment, as well as the inability of poly (A:U) to restore RFC in irradiated mice reconstituted with bone marrow cells only, argue against this possibility. It is important, however, that in experiments involving the use of thymic extracts and NTx animals attention be given to the fact that small numbers of residual thymic cells remain which might be stimulated to a remarkable degree by nucleic acids released on extraction of the thymus, acting like poly A:U to restore competency.

**SUMMARY**

Polyadenylic-polyuridylic acid complexes, a potent adjuvant to the immune response, were tested for action on thymic-influenced and bone marrow-derived lymphocytes in model systems deficient in one or the other of these cells.

Adult mice, thymectomized at birth or mice treated with heterologous antithymocyte serum produced 90–95% fewer splenic rosette-forming cells than normal mice in response to an injection of sheep erythrocytes. Intravenous injection of complexes of homoribopolynucleotides, polyadenylic and polyuridylic acids, poly A:U with SRBC restored immunologic competence to NTx- or ATS-treated mice such that they produced normal or near normal levels of splenic RFC. In addition, injection of poly A:U enabled NTx mice to reject allogeneic skin grafts at the same rate as control mice with an intact thymus. Further reduction in residual thymocytes by combining neonatal thymectomy with ATS treatment reduced the number of anti-SRBC RFC induced by poly A:U.

Lethally irradiated mice which received SRBC, excess bone marrow cells, and as few as 40,000 thymic lymphocytes were stimulated by poly A:U to produce RFC. No adjuvant effect was observed when irradiated mice received excess thymic lymphocytes and low doses of bone marrow cells with poly A:U.

The results suggested that the adjuvant action of poly A:U was exerted on the thymic-influenced, antigen-reactive cell and that restoration of immunocompetence to NTx- or ATS-treated mice was caused by amplification of a small number of residual antigen-reactive cells which were influenced by the thymus in utero before thymectomy, or which survived treatment with ATS.
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