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Mouse Thymocyte Membrane Uptake of Polyadenylic-Polyuridylic Acid Complexes (39651)

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Complexes of homoribopolymers such as polyadenylic acid and polyuridylic acid [poly(A:U)] are known stimulators of thymocyte activity in both cell-mediated immunity and humoral antibody formation (1). Although the mechanism of action is unknown, it has been suggested that the effects of poly (A:U) are mediated through the cell membrane (2). However, Fenster *et al.* (3) have shown that poly (A:U) rapidly penetrates certain mammalian cells and is soon located in the cell nuclei. Consequently, we have studied the uptake of poly (A:U) *in vitro* by mouse thymocytes utilizing indirect immunofluorescence. Evidence is presented which indicates that approximately 80% of thymocytes bind poly (A:U) on their membranes.

Materials and methods. Animals. Three-month-old New Zealand white male rabbits were used for formation of antibody to poly (A:U). Balb/Aj male and female mice inbred in our laboratory were used throughout.

Preparation of antisera. Poly (A) (Lot No. 77 Code 11-301), poly (U) (lot No. 72 Code 11-308), and methylated bovine serum albumin (MBSA lot 15) were obtained from Miles Laboratories, Inc., Elkhart, Ind. Complete Freund's adjuvant was obtained from Difco, Co., Detroit, Michigan. The polymers were prepared at a concentration of 5 mg/ml in 0.15 *M* NaCl and mixed to form the helical polymer, poly (A:U). These solutions were refrigerated at 4°.

Dilutions were made in 0.15 *M* NaCl to a concentration of 1 mg/ml. MBSA was prepared in a 1% aqueous solution. Equal volumes of the two solutions were mixed, and the complex was emulsified with an equal volume of Freund's complete adjuvant to a final concentration of 454 μg of antigen/ml. Fresh antigen was prepared at the time of each injection.

Rabbits were injected intramuscularly with 1.4 ml of the antigen preparation in divided doses, weekly for 4 weeks. The final injection, administered in the fifth week, did not contain Freund's adjuvant. The animals were bled 8 days following the last injection.

Sera were heat inactivated and absorbed four times using BALB/Aj spleen and thymocyte cells at a ratio of 1 vol of cells to 3 vol of sera. The sera were filtered and diluted 1:16 with 0.01 *M* PBS, pH 7.2, and frozen at −20°.

Ouchterlony double diffusion was used to evaluate the specificity of the antisera to poly (A:U). Precipitation was found at all concentrations of poly (A:U) tested: 30, 125, 250, and 1000 μg/ml. No precipitation was seen with several concentrations of BSA or MBSA, nor with poly (A) or poly (U) alone.

A commercial preparation of fluorescein isothiocyanate conjugated (FITC) goat anti-rabbit immunoglobulin (IgG, IgA, IgM), from Behring Diagnostics (Lot F458AE, Molar F/P ratio 3.0), was used. The FITC goat antiserum was absorbed twice using Balb/Aj thymocyte and spleen cells, diluted 1:3 with 0.01 *M* PBS, and stored at −20°. DEAE-dextran was obtained from Pharmacia Fine Chemicals Inc., Piscataway, N.J.

Cell suspensions. Following cervical dislocation, thymuses were excised from 4- to 6-week-old Balb/Aj mice and placed in modified salt solution (MSS). The tissues were teased, the suspensions were allowed to sediment, and the supernatant was removed and centrifuged at 1000 rpm for 10 min at 4°. The cells were washed twice with MSS and resuspended in Veronal buffered solution (VBS), pH 7.2, containing 10% heat-inactivated fetal calf serum (FCS). The cells were suspended at a count of 1.8–2.1 × 10^7 cells/ml.

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Aliquots of 0.2 ml of thymocyte cell suspension were placed into small glass tubes. One-tenth of a milliliter of poly (A:U), 500 μg/ml, was added to the tube, and the suspension was incubated at 4° for 10 min. The suspension was then centrifuged for 6 min at 900 rpm at 4°, and the supernatant was discarded. The suspension was washed twice in an excess of VBS plus 10% FCS. After the cells were pelleted, 0.10 ml of a 1:16 dilution of antiserum to poly (A:U) was added to the tube. The mixture was incubated for 10 min at 4°, centrifuged as described, and then washed twice in excess VBS plus 10% FCS. Following pelleting, 0.02 ml of FITC goat anti-rabbit immunoglobulin was added to the tubes and incubated for 10 min. The mixture was centrifuged and washed twice with excess VBS plus 10% FCS.

Immunofluorescence. A Carl Zeiss fluorescence microscope was used. The cell suspensions were examined under ultraviolet light at a magnification of 400×, and at least 200 cells were counted per sample.

Controls. Each experiment included a control cell suspension to which poly (A:U) was not added. In addition, control samples with and without poly (A:U) were examined in which the serum of a normal nonimmunized rabbit, diluted 1:16 and absorbed as described, was substituted for the antiserum to poly (A:U).

Results. Uptake of poly (A:U) by thymocytes. A mean of 80 ± 4% thymocytes exhibited membrane-bound poly (A:U) in six experiments. The patterns of staining observed included ring formation, speckling, and capping (Fig. 1). Those cells displaying a homogeneous stain were not counted and were considered nonviable. The percentage of fluorescent cells in any control sample never exceeded 3%. Cell viability consistently exceeded 95% as determined by trypan blue dye exclusion.

The data of Table I show that the uptake of poly (A:U) by thymocytes increased as the concentration of (A:U) increased. Only 6% of cells stained at a concentration of 5 μg/ml of poly (A:U), whereas 44% of the thymocytes stained at a concentration of 50 μg/ml. A maximum of 84% of cells stained at 500 μg/ml; this percentage was not raised by a 10-fold increase in concentration.

When thymocytes were preincubated with either poly (A) alone, 500 μg/ml, or poly (U) alone, 500 μg/ml, and then washed, and incubated with poly (A:U), there was no decrease in the number of stained cells nor any alteration in the pattern of the stain.

In order to ascertain what effect the duration and temperature of incubation would have on the degree of fluorescence uptake by thymocytes, the cell suspensions were incubated for 1, 10, 30, and 60 min at either 4 or 37°. The remaining steps of the procedure were conducted at 4°. As indicated in Table II, the temperature at which incuba-

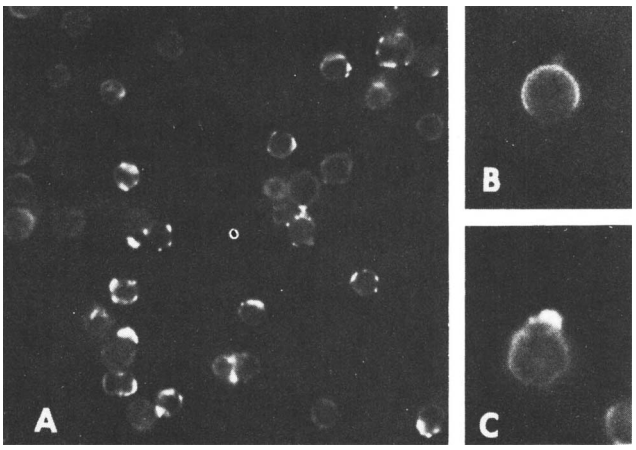

FIG. 1. Microphotograph of fluorescent thymocytes. (A) Field of thymocytes previously incubated with poly (A:U) and rabbit antisera to poly (A:U) then counterstained with goat FITC anti-rabbit Ig. Observe the speckled pattern. (B) Ring pattern. (C) Capping. (400×).

tion was carried out as well as the duration of incubation had little effect on the membrane uptake of poly (A:U) by the thymocytes. A repeat of this experiment yielded essentially the same data.

Uptake of poly (A:U) by cortisone-resistant thymocytes. The uptake of poly (A:U) by the thymocytes of steroid-treated mice was examined. One-month-old Balb/Aj mice were injected intraperitoneally with 2.5 mg of hydrocortisone succinate on 2 consecutive days. The thymuses were removed on the third day, and cell suspensions were obtained in the usual manner. The number of cells demonstrating fluorescent staining was reduced markedly in hydrocortisone-treated mice in contrast to the thymocytes taken from noninjected control littermate mice (Fig. 2).

Inasmuch as steroids are known to stabilize the membranes of cells, a further experiment was conducted to determine if steroid uptake or coating of the membrane may have been responsible for the reduction in fluorescein uptake rather than the removal of steroid-sensitive thymocytes. Accordingly, thymocytes from normal control mice were incubated in VBS plus 10% FCS or, in this medium plus 100 μg/ml of hydrocortisone succinate for 20 min prior to incubation with poly (A:U). There was no difference in the uptakes of fluorescein stain between nonsteroid-treated cells (68%) and steroid-treated cells (60%). In addition, the degree of fluorescein uptake was not influenced by a 10-fold higher concentration of hydrocortisone.

Influence of DEAE-dextran on uptake of poly (A:U). Penetration of FLS Swiss mouse ascites tumor cells and human lymphocytes by poly (A:U) has been shown to increase 20-fold when the cells are pretreated with DEAE-dextran (3). In an attempt to ascertain whether DEAE-dextran pretreatment would increase the number of fluorescent cells or alter the pattern of staining of mouse thymocytes, the following experiments were conducted. In the first, thymocytes were pretreated for 15 min with DEAE-dextran at either 20 or 200 μg/ml, and then washed several times before poly (A:U) was added. Thymocytes from the same cell suspension, not pretreated with DEAE-dextran, were used as controls.

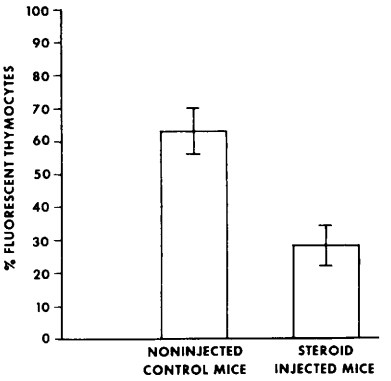

FIG. 2. Alteration in the number of fluorescent thymocytes in mice injected with hydrocortisone succinate. The two bars represent the mean of four paired experiments. The bar on the left indicates that 63 ± 7% of the thymocytes taken from uninjected control littermate mice showed fluorescence. The bar on the right reveals that 28 ± 6% of the thymocytes from steroid-injected mice showed fluorescence. This difference was significant using Student's paired *t* test ($P < 0.005$).

TABLE I. CORRELATION OF POLY (A:U) CONCENTRATION AND NUMBER OF FLUORESCENT THYMOCYTES.

Concentration of poly (A:U)[a] (μg/ml)	Fluorescent thymocytes (%)
0	3
5	6
50	44
500	84
5000	76

[a] Concentration of poly (A:U) in 0.1 ml added to 0.2 ml of a 1.8–2.1 × 10^7 cells/ml of thymocyte cell suspension.

TABLE II. EFFECT OF TEMPERATURE AND DURATION OF INCUBATION ON NUMBER OF FLUORESCENT THYMOCYTES.[a]

Incubation time (min)	Temperature (°C)	Fluorescent thymocytes (%)
1	4	67
1	37	78
10	4	81
10	37	72
30	4	74
30	37	78
60	4	78
60	37	71

[a] In each experiment, 0.1 ml of poly (A:U), 500 μg/ml, was added to a 0.2-ml aliquot of thymocyte cell suspension 1.8–2.1 × 10^7 cells/ml.

There was no significant difference in the number of positive-staining cells between the two sets of cells (no pretreatment, 74%; pretreatment with 20 μg/ml of DEAE-dextran, 80%; pretreatment with 200 μg/ml of DEAE-dextran, 70%). The morphologic appearance of the fluorescent cells was identical to those fluorescent thymocytes not pretreated with DEAE-dextran.

As illustrated in Table III, addition of DEAE-dextran and poly (A:U), simultaneously, reduced the number of positive cells. It would appear that the positively charged DEAE-dextran may have reacted with the negatively charged poly (A:U), precluding uptake by the thymocytes.

Capping. As mentioned earlier, the poly (A:U), anti-poly (A:U) complex was found to cap when incubation was carried out at 37°. In order to quantitate the frequency of capping of the thymocytes, these cells were incubated at 4 or 37° in RPMI-1640 for periods of 2 and 2.5 hr. Examination of the cells incubated at 4° for either time period revealed the same percentage of stained cells and pattern of staining. Minimal capping occurred at 4°. On the other hand, cells incubated at 37° uniformly exhibited capping. In addition, after incubation, the number of stained cells decreased from 72 to 57% at 2 hr, and to 39% at 2.5 hr (Table IV).

TABLE III. EFFECT OF COMBINED DEAE-DEXTRAN AND POLY (A:U) INCUBATION ON THE NUMBER OF FLUORESCENT THYMOCYTES.

Expt	DEAE-dextran[a] 200 μg/ml	DEAE-dextran[a] 20 μg/ml	Poly (A:U) 500 μg/ml	Fluorescent thymocytes (%)
1	+	−	+	10
	−	+	+	30
	−	−	+[b]	79
	−	−	−[c]	3
2	+	−	+	11
	−	+	+	25
	−	−	+	69
	−	−	−	2

[a] The concentrations of DEAE-dextran used when 0.1 ml was added together with 0.1 ml of poly (A:U), to 0.1 ml of thymocyte cell suspension, 3.6–4.0 × 10^7 cells/ml for a total volume of 0.3 ml.

[b] Here, 0.1 ml of VBS was added in place of DEAE-dextran to 0.1 ml of poly A:U and 0.1 ml of thymocyte cell suspension, 3.6–4.0 × 10^7 cells/ml.

[c] A total of 0.2 ml of VBS was added to 0.1 ml of thymocyte cell suspension.

Discussion. Cells responding to the adjuvant action of poly (A:U) include the macrophage, T and B lymphocytes, stem cells, and memory cells (1). In addition, nonimmunocompetent cells such as those in the parotid gland and pancreas have demonstrated enhanced function under the influence of those polynucleotides (1). Inasmuch as the immune response to low numbers of thymocytes was enhanced remarkably by poly (A:U) (4), the binding of the adjuvant to these cells was measured in this initial study. The data show that the majority of mouse thymocytes possess membrane receptor sites for poly (A:U), and that a subpopulation of cells, presumably the more mature medullary thymocytes, bind poly (A:U) to a much lesser degree than the cortical thymocytes. DEAE-dextran appeared not to enhance or induce membrane staining of those thymocytes which by themselves did not bind poly (A:U).

In previous studies in our laboratory, the steroid-resistant (medullary) thymocyte has been implicated as one of the cells involved in the expression of the adjuvant action of polynucleotide complexes (5). In addition, steroid-resistant thymocytes have been shown by others to be fully capable of immunological response despite marked diminution in numbers of steroid-sensitive (cortical) cells (6). However, in this investigation, only 20–30% of thymocytes from steroid-treated mice revealed the presence of poly (A:U) on the membrane surface. This apparent paradox might be explained with the assumption that poly (A:U) may pene-

TABLE IV. INFLUENCE OF TIME AND TEMPERATURE ON CAPPING OF POLY (A:U) ON THYMOCYTES

Duration of incubation after fluorescein staining (min)	Temperature (°C)	Percentage of fluorescent thymocytes: Speckled, ring	Cap	Total
0	4	77	4	81
120	4	72	6	78
150	4	74	5	79
0	37	69	3	72
120	37	4	53	57
150	37	3	36	39

trate the medullary thymocyte membrane via endocytosis without an intermediary stage of attachment to the membrane itself. Schell (7) showed that poly (A:U) entered the Ehrlich ascites tumor cell without separation of base pairing or breaking of the strands. Confirmatory work by Fenster *et al.* (3) indicated that poly (A:U) was rapidly taken into human lymphocytes and mouse ascites tumor cells and could be found as intact double-strand complexes in the nucleus and cytoplasm. In contrast, poly (A:U) may attach to membrane receptor sites available on cortical thymocytes with ingestion occurring over a longer period of time. If this hypothesis is correct, then, adjuvant activity ought to be associated with penetration of the plasma membrane by the complex.

The immunofluorescent technique used in these experiments detected the presence of poly (A:U) on the membrane only, and not intracellularly. Previous attempts to study the fate of poly (A:U) on cells by Schell (7, 8) and Fenster *et al.* (3) utilized isotopic techniques which enabled them to trace the fate of poly (A:U) intracellularly.

Fenster *et al.* (3) demonstrated that DEAE-dextran-pretreated ascites tumor cells and human lymphocytes increased penetration by poly (A:U) by 20-fold. In the present study, DEAE-dextran caused no increase in the number of thymocytes with membrane-detectable poly (A:U). Thus, these contrasting results suggest that membrane penetration of the medullary thymocyte may be enhanced by DEAE-dextran without requiring initial receptor site attachment to the membrane. Accordingly, medullary thymocyte membranes, by virtue of the maturation process, may demonstrate membrane dynamics that differ from the cortical thymocytes, i.e., the presumed blastic changes exhibited by medullary thymocytes and subsequent membrane alterations.

The present investigation would suggest that there may be at least two mechanisms of cellular uptake by mouse thymocytes of poly (A:U). One would involve a rapid penetration through the membrane by the double-stranded complex, as in the study of Fenster *et al.* (3), and another, by adsorption to the receptor sites on the membranes of different cell types with subsequent membrane activation, capping, and ingestion by the cell. These receptors appeared specific for double-stranded polynucleotides in that they could not be blocked by single stranded poly (A) or poly (U).

Preliminary studies indicate that Balb/Aj peritoneal exudate cells, rat liver cells, and human peripheral T lymphocytes do not have surface receptors for poly (A:U).

Summary. Indirect immunofluorescence revealed that 80 ± 4% of isolated mouse thymocytes were found to have membrane uptake of poly (A:U). Mice previously injected with hydrocortisone demonstrated a reduction to 20–30% in numbers of fluorescent thymocytes. Preincubation of thymocytes with DEAE-dextran did not increase the percentage of fluorescent cells.

These findings suggest that medullary thymocytes bind poly (A:U) to a lesser degree than cortical thymocytes. Several mechanisms of thymocyte uptake of poly (A:U) are suggested including adsorption to the membrane and/or rapid penetration through the membrane.

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