RESPONSES TO IMMUNIZATION IN THE THYMUS OF THE ADULT MOUSE*

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Two classes of cells may be involved in the sequence of events which begins with the injection of antigen into the immunocompetent host and ends with the appearance of circulating antibody. Neonatally thymectomized, X-irradiated mice who received either thymocytes, or bone marrow formed significantly less antibody than mice receiving both classes of cells together (1, 2). Thymus cells did not form antibody in the intact animal (3, 4). Nossal et al. found (5) that all the antibody-forming cells in the fully reconstituted host were derived from the bone marrow. Thus, thymus-derived cells appeared to play a indirect role in the formation of antibody by bone marrow cells.

Cells in the interfollicular cortex of the mouse spleen appeared to be of thymic origin (6). Members of this population underwent division after an injection of sheep red blood cells (SRBC) and, although they did not produce antibody were nevertheless antigen sensitive (7, 8).

It may be that cells in the thymus of the adult mouse responded to antigen as well. Since such cells do not form antibody (2), we chose another parameter of response, namely, the biosynthesis of RNA. After the injection of SRBC, we found that the rate of RNA synthesis increased in the thymus. A material was present in extracts of RNA from the thymus of immunized mice which specifically converted a small proportion of either peritoneal cells or spleen cells from nonimmunized mice to form antibody.

Materials and Methods

Animals and Immunization.—CD-1 female mice (Charles River Breeding Laboratories, North Wilmington, Mass.), 10–16 wk old were used throughout. They were immunized by the intraperitoneal injection (i.p.) of 0.15 ml of a 25% suspension of washed sheep red blood cells (SRBC) 3 days before they were killed. Experimental and control mice were kept in air-conditioned animal rooms and fed Purina mouse chow and water ad libitum.

Extraction of RNA and Separation by Density Gradient Centrifugation.—RNA was ex-

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tracted from either the thymus glands or spleens of immunized or control mice by the hot phenol method (9) as described previously (10). Chromatographic grade phenol (Mallinkrodt Chemical Works, St. Louis, Mo.), without preservative was redistilled at frequent intervals. The RNA preparations were separated by centrifugation in a SW 25.1 rotor for 18 hr at 23,000 rpm in a Spinco model L-2 ultracentrifuge. Fractions were collected from the bottom of the tube and the optical density at 260 mμ was determined.

Preparation of Cells and Radioactive RNA.—Suspensions of either thymus or spleen cells were prepared by gently forcing the tissue through a number 40 stainless steel screen into cold Eagle's medium (Baltimore Biological Laboratories, Baltimore, Md.). The cells were dispersed further by gentle pipetting in the cold. Cellular RNA was labeled with tritium during a 60 min incubation with shaking at 37°C in Eagle's medium containing 10 μc/ml tritium-labeled uridine (Schwarz BioResearch, Inc., Orangeburg, N.Y., specific activity approximately 20c/mM). The volume of cells and medium was kept rather small, usually no more than 10 ml medium for the cells from 20 glands. RNA was extracted from both cells and incubation medium by the addition of the phenol solution directly to the mixture, followed by the usual separation procedure.

Tests of RNA for Biological Activity.—RNA was tested for its capacity to convert normal cells to antibody-forming cells as described previously (10, 11). In brief, approximately 1.0 ml of thymic RNA in medium 199 (Baltimore Biological Laboratories) was added to 2.0 ml of a suspension of spleen cells from nonimmunized mice in a 25 ml Erlenmeyer flask. The stoppered flask was incubated for 5 min at 37°C in a water bath equipped with a shaking device. After incubation, the proportion of cells forming antibody to SRBC was determined by the plaque assay method (12). Tubes containing 2.0 ml of 0.7% agarose (L'Industrie Biologique Francaise S. A., Gennevilliers, France) in medium 199, prepared just before use, were maintained at 45°C and cooled for approximately 30 sec at room temperature before the addition of 0.1 ml of a 10% suspension of SRBC, followed by 0.4 ml of the RNA-cell suspension. The mixture was mixed quickly and spread over warm 100 ml Petri plates which contained a 1.4% agar base. The plates were incubated for 2 hr at 37°C, after which 1.5 ml of at least a 33% solution of freshly prepared guinea pig serum was added. Incubation for an additional 1 hr at 37°C and at 4°C overnight was carried out before the plates were examined under low power magnification. Each plaque was symmetrically round and had an identifiable cell in its center.

Determination of Specific Activity of RNA.—To determine the specific activity of individual fractions of RNA the optical density was determined and then the RNA was coprecipitated with 100 μg of carrier yeast RNA by adding enough 50% trichloroacetic acid to make a final 5% solution. After incubation at 4°C, overnight, the precipitates were collected on nitrocellulose filters which were then dried and the radioactivity which remained was determined. Optical density of 0.030 = 1.0 μg RNA.

Preparation of Rabbit Antithymus Serum.—Thymus glands from 10 CD-1 mice were homogenized in 10 ml of medium 199 in a glass-teflon homogenizer and then emulsified in an equal volume of incomplete Freunds adjuvant. Adult female rabbits were injected in four subcutaneous sites, followed in 2 wk by a second injection of homogenized thymus without adjuvant. 2 wk after the second injection, the rabbits were bled from the marginal ear vein. The serum titer, as determined by complement-mediated cytotoxicity toward a suspension of mouse thymus cells was approximately 1:96.

Polyacrylamide Gel Electrophoresis of RNA.—Polyacrylamide gels, for electrophoresis of RNA, were prepared as described by Weinberg et al. (13). Freshly recystallized acrylamide and *N*,*N*-[1]-methylene-bisacrylamide (Eastman Kodak Co., Rochester, N.Y.) were mixed in a Tris-acetate buffer at pH 7.4 and polymerized by the addition of ammonium persulfate and tetramethylethylene diamine. The final concentration of the acrylamide gel was 3.5%. Im-

mediately after the addition of the catalysts, the mixture was poured into 0.4 × 6 cm tubes and placed in a gel electrophoresis apparatus (Canal Industrial Corp., Bethesda, Md.). Separations took place at room temperature during a 90 min run at 5 ma per gel. RNA was eluted from 4 to 6 mm slices of the gel and the specific activity of each fraction was determined.

RESULTS

1. RNA Synthesis in the Thymus after Immunization

(*a*) *Specific Activity of RNA from Thymus Glands of Adult Mice after Immunization: Sucrose Gradient.*—Mice were injected i.p. with SRBC. At varying times thereafter, their thymus glands were removed and a suspension of thymocytes was prepared. The cells (greater than 95% viable) were incubated at 37°C for 60 min in Eagle's medium containing tritium-labeled uridine. After incubation, RNA was extracted from both cells and incubation medium and separated in a density gradient. The specific activity of each fraction was determined. The results (Fig. 1) indicated that after immunization, cells from the adult thymus synthesized RNA faster than control, nonimmunized mice. A new peak of RNA of greater specific activity was found 1 day after antigen injection at that part of the gradient which corresponded to approximately 8–12S. By 14 days the pattern had returned to control levels.

A large proportion of the RNA was of small molecular weight. If the extraction was performed on the cells without the incubation medium, the characteristic three peaks of RNA were observed.

(*b*) *Effect of Rabbit Anti-Mouse Thymus Antiserum on the Response of the Thymus of the Adult Mouse to Immunization: Sucrose Gradient.*—Mice were injected i.p. with SRBC and 0.1 ml of rabbit anti-mouse thymus sera. At varying times thereafter, the thymus glands were removed and a suspension of thymus cells was incubated with tritium-labeled uridine as described above. The labeled RNA was separated in a density gradient and the specific activity of each fraction was determined as before. The results presented in Fig. 2, indicate a large reduction in the rate of RNA synthesis had occurred in animals that received anti-thymus serum. The effect was observed only after 24 hr and persisted for at least 11 days. Grossly, the glands were smaller than controls, although by 11 days they had returned to approximately normal size.

(*c*) *Further Separation of RNA from Thymus Glands of Adult Mice: Polyacrilamide Gel Electrophoresis.*—CD-1 mice were injected i.p. with SRBC and, as before, cellular RNA from their thymus glands was labeled with tritium during a 1 hr incubation in vitro. The RNA was separated in a density gradient. The gradient was divided into three portions, corresponding to the area under the upper or top, middle, and lower one-thirds. The fractions from each third were combined and the RNA's were precipitated and resolubilized in buffer. Then the RNA was separated further by polyacrilamide gel electrophoresis. After electrophoresis, the gels were sliced into 4–6 mm pieces (about 10–15

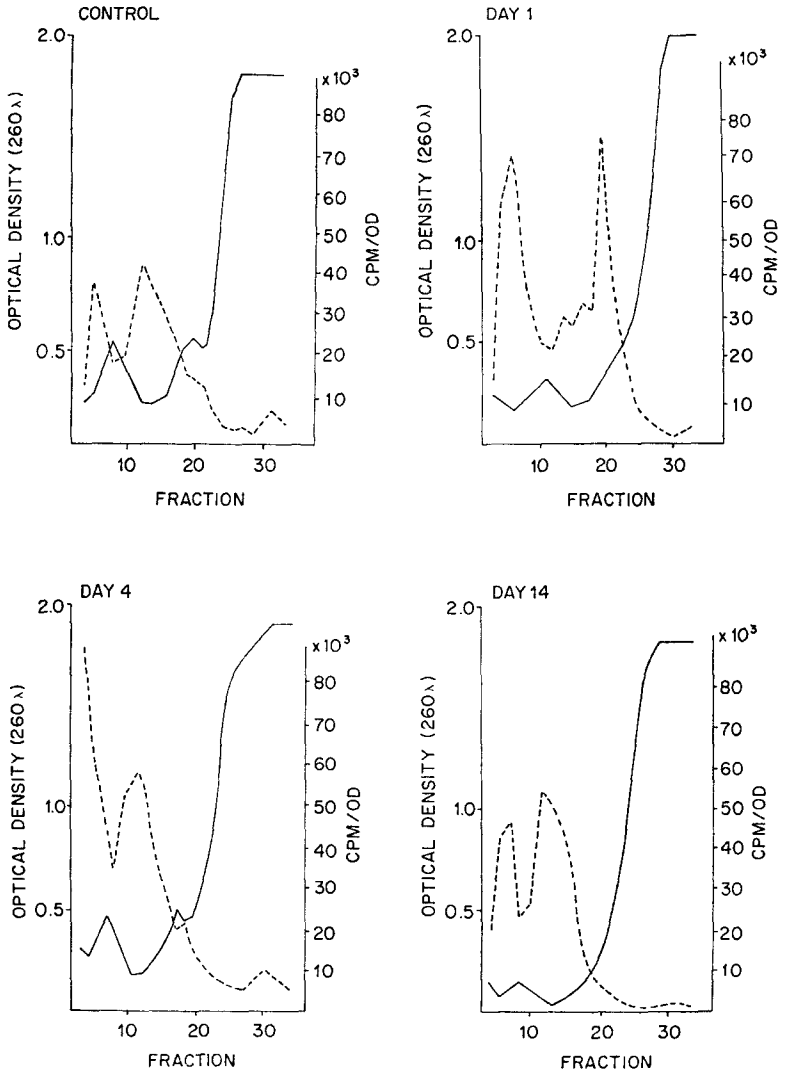

FIG. 1. The effect of immunization with SRBC on RNA synthesis in the mouse thymus after immunization. Sucrose gradient. Adult CD-1 mice were injected with SRBC. Control mice were not injected. At varying times thereafter, suspensions of cells from their thymus glands were incubated 60 min in a medium containing tritium-labeled uridine. After incubation, RNA was extracted from both cells and incubation medium and then separated by density gradient in sucrose. The specific activities of the various fractions were determined. Extraction of RNA from cells alone yielded the usual three peaks of OD. Solid line: optical density. Dashed line: cpm/OD.

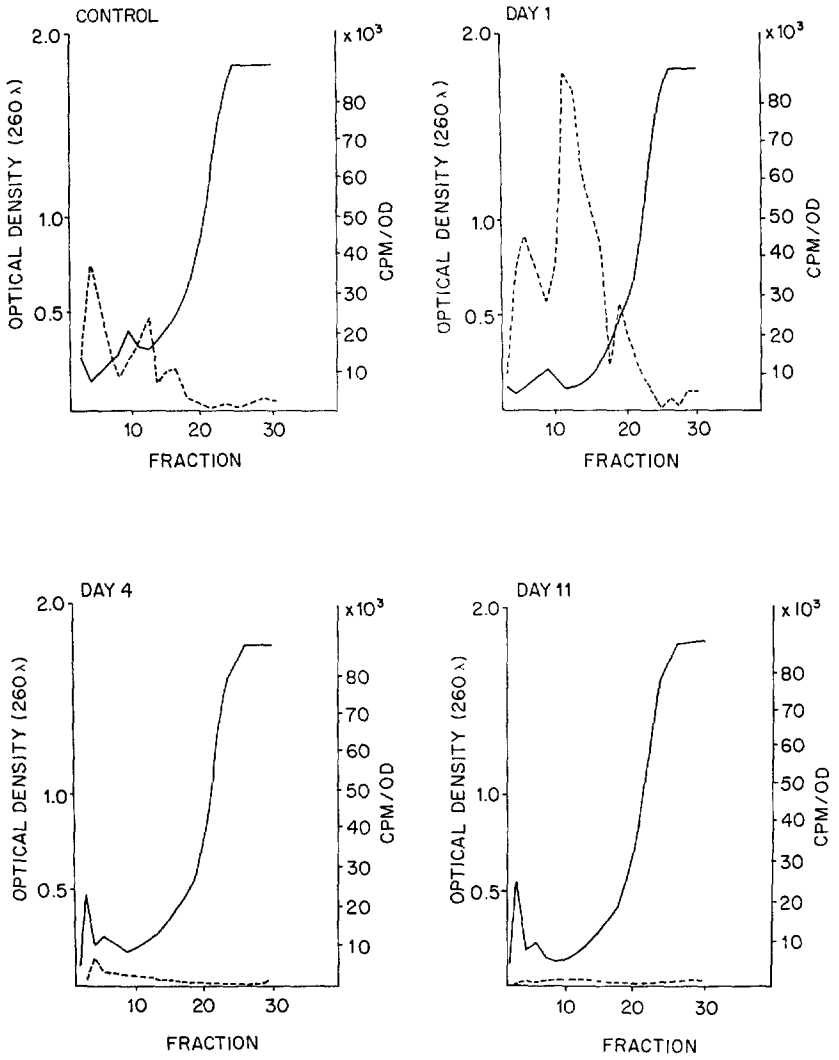

FIG. 2. The effect of anti-mouse thymus serum on RNA synthesis in the thymus after immunization. Sucrose gradient. Adult CD-1 mice were injected with SRBC and 0.1 ml of rabbit anti-mouse thymus serum, or were not injected. At varying times thereafter, suspensions of cells from their thymus glands were incubated 60 min in a medium containing tritium-labeled uridine. After incubation, RNA was extracted from both cells and incubation medium and then separated in density gradients. The specific activities of the various fractions were determined. Solid line: optical density. Dashed line: cpm/OD.

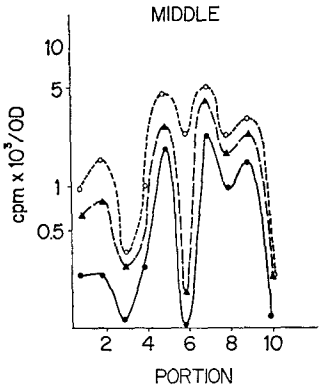

FIG. 3. The effect of immunization on RNA synthesis in the thymus. First polyacrylamide gel electrophoresis. Adult CD-1 mice were injected with 0.1 ml of a 25% suspension of SRBC or were not injected. At varying times thereafter, suspensions of cells from their thymus glands were incubated 60 min in a medium containing tritium-labeled uridine. After incubation RNA was extracted from both cells and incubation medium and then separated by density gradient centrifugation. The RNA from the middle one-third of the gradient was separated further by polyacrylamide gel electrophoresis and the specific activity of the various fractions determined. First slice from the top of the gel. Solid line: control. Dashed line: 2 days. Dotted line: 3 days.

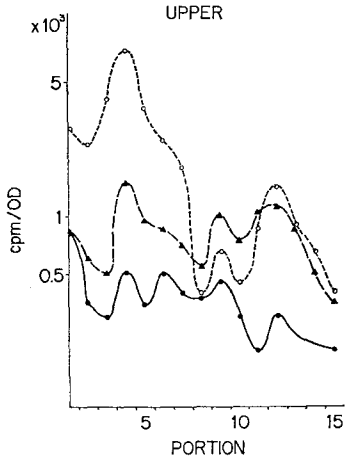

FIG. 4. The effect of immunization on RNA synthesis in the thymus after immunization. Second polyacrylamide gel electrophoresis. The conditions of the experiment were the same as in Fig. 3 except that the RNA from the upper one-third of the gradient was separated further by polyacrylamide gel electrophoresis. Solid line: control. Dashed line: 2 days. Dotted line: 3 days.

slices) and the RNA from each slice was eluted and its specific activity was determined. The results, (Figs. 3 and 4) reveal that RNA's from the thymus glands of immunized mice were of higher specific activity than RNA's from control mice. The rapidly labeled RNA's were distributed throughout the gel. Analysis of the pattern of labeling of the middle one-third of the gradient (Fig. 3) corresponding to approximately 8–20S, showed two main peaks and a progressive rate of labeling over a 3 day period. Some fractions from gel electrophoresis of RNA's from the upper one-third of the sucrose gradient showed a 16-fold increase in specific activity (Fig. 4).

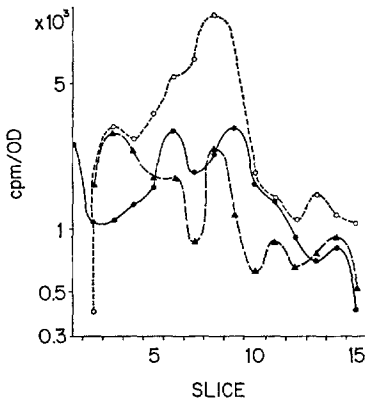

FIG. 5. The effect of antimouse thymus serum on RNA synthesis in the thymus after immunization. Polyacrylamide gel electrophoresis. Adult CD-1 mice were injected with either SRBC, SRBC and 0.1 ml of rabbit anti-mouse thymus serum or were not injected. On day 3 after injection, suspensions of cells from their thymus glands were incubated 60 min in a medium containing tritium-labeled uridine. After incubation, RNA was extracted from both cells and incubation medium and then separated by density gradients in sucrose. RNA from the upper one-third of the gradient was separated further in polyacrylamide gel electrophoresis. Solid line: not injected. Dashed line: injected with SRBC and anti-thymus serum. Dotted line: injected with SRBC alone.

d. Effect of Rabbit Anti-thymus Serum on the Labeling of RNA of the Immunized Adult Mouse Thymus. Polyacrylamide Gel Electrophoresis.—Antimouse thymus serum was prepared as described above. Adult mice received SRBC and 0.1 ml of antiserum i.p. 3 days later, their thymus glands were removed and a suspension of thymus cells was incubated in vitro for 60 min in medium containing tritium-labeled uridine. Control animals received SRBC without antiserum. Other control mice were not injected.

Antithymus serum markedly affected the synthesis of RNA in mice injected with SRBC (Fig. 5). Mice that received SRBC plus antiserum showed a reduced rate of incorporation of label into RNA.

TABLE I

Biological Activity of RNA-Extracts from the Thymus

Experiment	RNA		Cells		Background plaques	No. plaques> background
	Source	Quantity	Source	Quantity		
		μg		$\times 10^6$		
1	Thymus	500	p.e.*	30	95	101
2	Thymus	40	Spleen	25	19	17
	Spleen	40	"	25	19	16
3	Thymus	400	p.e.	63	18	70
	Spleen	2300	"	63	18	59
4	Thymus	83	p.e.	13	192	87
	Thymus after RNAse	83	p.e.	13	192	0
5	Thymus	700	p.e.	40	29	11
	Thymus after RNAse	700		40	29	0
6	Thymus	176	p.e.	15	12	13
	Thymus after RNAse	176		15	12	0
7	Thymus	1200	Spleen	400	109	41
	Thymus after RNAse	1200		400	109	0
8	Thymus	500	Spleen	40	31	35
	"	500	Thymus	40	2	0
	Spleen	2000	Spleen	40	31	52
	"	2000	Thymus	40	5	0
9	Thymus	1200	Spleen	133	47	33
	"	1200	Thymus	107	4	3
	Spleen	4000	Spleen	133	48	77
	"	4000	Thymus	107	4	4
	Liver	4380	Spleen	133	45	0
10	Thymus	292	Spleen	352	5	10
	N-thymus‡	1000	"	352	4	0
	Spleen	1330	"	352	5	14
11	Thymus	160	Spleen	85	28	0
	"	320	"	85	28	29
	"	480	"	85	28	30
	N-thymus‡	158	"	85	18	0
	"	317	"	85	32	3
	"	475	"	85	16	0
12	Thymus	15	Spleen	33	3	10
	Liver	15	"	33	3	3
	Spleen	15	"	33	3	10

TABLE I—*Continued*

Experiment	RNA		Cells		Background plaques	No. plaques> background
	Source	Quantity	Source	Quantity		
		μg		×10⁶		
13	Thumus	117	p.e.	60	14	26
	Spleen	700	"	60	13	40
	Liver	765	"	60	14	3
14	Thymus	1710	Spleen	313	33	17
	Thymus-RRBC§	1710	"	313	26	0
15	Thymus	810	Spleen	225	31	16
	Thymus-RRBC§	810	"	225	24	0
16	Thymus	975	Spleen	100	7	7
	"	975	"	100	0	0 (rabbit)‖
17	Thymus	522	Spleen	100	20	17
	Thymus-RRBC§	522	"	100	15	0
	"	1044	"	100	20	5
18	Thymus	572	Spleen	110	42	45
	"	572	"	110	0	0 (rabbit)‖
	Thymus-RRBC§	572	"	110	27	0

The results of 18 separate experiments are presented. Approximately 20 CD-1 mice were injected i.p. with SRBC 3 days before they were killed. RNA was extracted by the hot phenol method from either the spleen, thymus or liver as indicated.

Cells were obtained from either the spleens or peritoneal cavities of CD-1 mice not injected with SRBC. After the cells were incubated with RNA for 5 min at 37°C, the proportion forming antibody to SRBC was determined by the plaque assay method. In each experiment, fractions were taken from a pool of cells. Variation between duplicate background determinations was less than 10%.

In those experiments involving degradation of RNA with ribonuclease, digestion was carried out at 37°C for 30 min at an enzyme:substrate ratio of 1:100.

* p.e., peritoneal exudate.

‡ RNA from glands of normal mice.

§ Immunized with RRBC.

‖ Indicator RBC.

2. *Conversion of Nonimmune Cells to Antibody-Forming Cells with an RNA Extract from the Thymus*

RNA extracts were prepared from the thymus glands of mice injected 3 days previously with SRBC. Control extracts were prepared from either the spleens or livers of mice injected 3 days previously with SRBC or from the thymus glands of mice not injected. RNA was added to suspensions of cells from either the spleens, peritoneal cavities, or thymus glands of normal mice, after which the proportion of cells forming sheep cell hemolysins was determined by the

plaque assay method. The results of 18 experiments are presented in Table I. In each experiment, a separate extraction was performed; fractions from a single pool of cells were removed for exposure to the extracts. The variation between duplicate "background" assay plates was less than 10%. RNA extracts from the thymus glands or spleens of immunized mice converted significant proportions of normal cells to cells which released sheep cell hemolysins. Extracts from the thymus glands of nonimmunized mice as well as the livers of

TABLE II

Activity of RNA Extracts Separated in Gel Electrophoresis

RNA source	Experiment	
	1*	2
Spleen:		
upper	0	0
middle	11	48
lower	1	0
Thymus:		
upper	8	46
middle	0	0
lower	12	21

RNA first was extracted from the spleens and thymus glands of CD-1 mice that were injected with SRBC 3 days previously and then sedimented in a sucrose gradient. The gradients were divided into three groups corresponding to upper, middle, and lower thirds, and the RNA was precipitated from each with 2 volumes of absolute ethanol. RNA from the upper third was resolubilized in buffer and further separated by polyacrylamide gel electrophoresis.

After electrophoresis, the gel was cut into thirds. RNA, eluted from each third, was added to portions of a suspension of cells from the spleens of CD-1 mice not injected with SRBC. After incubation, the proportion of cells forming antibodies to SRBC was determined by the plaque assay method.

*Plaques greater than background.

immunized mice were without activity (Table I). The active material was sensitive to ribonuclease, although residual antigen or antigenic derivative which might remain was not determined. The reaction was specific because extracts from the glands of mice immunized with rabbit red blood cells (RRBC) failed to convert normal cells to form antibodies to SRBC.

3. *Conversion of Nonimmune Cells to Antibody-Forming Cells with either Thymus or Spleen Extracts Separated on Polyacrylamide Gel Electrophoresis*

Mice were immunized with SRBC. RNA extracts from their thymus glands were separated in sucrose density gradients. The upper one-third of the gradients were pooled and the extracts from these portions were separated further by

polyacrylamide gel electrophoresis. A suspension of spleen cells from nonimmunized mice was prepared and portions were incubated with RNA-extracts from the gels. After incubation, the proportion of cells forming antibodies to SRBC was determined by the plaque assay method. The results, (Table II) indicate that the RNA extracts from the thymus which contained biological activity were electrophoretically distinguishable from the extracts from the spleen which contained biological activity. The upper and lower portions of the gel containing RNA extracts from the thymus were active. The middle portion of the gel, containing extracts from the spleen alone, was active. RNA extracts from other portions of the gradient were inactive.

DISCUSSION

The thymus in the adult mouse responded to the injection of SRBC in at least several ways. The evidence presented in this report may be summarized as follows:

1. The rate of RNA synthesis in the thymus of 8–12 wk-old mice increased within 24 hr after the intraperitoneal injection of approximately 10^8 SRBC. After the RNA was sedimented in a density gradient of sucrose, two main peaks were observed. The first corresponded to RNA's whose sedimentation coefficient was greater than 28S; the second corresponded to RNA's whose sedimentation coefficient was approximately 8–12S. The effect persisted through 8 days. Rabbit anti-mouse thymus serum, injected along with SRBC into mice, inhibited RNA synthesis in the thymus. The inhibition was observed after day 1 and recovery had not occurred by day 11.

2. Further separation of RNA's of lower molecular weight by polyacrylamide gel electrophoresis showed that the 8–12S peak was in reality two peaks of apidly synthesized RNA and again the effect of antigen on RNA synthesis n the thymus was reversed by the simultaneous injection of antithymus serum.

3. Extracts of RNA from the thymus glands of mice immunized with SRBC converted a small proportion of either spleen cells or peritoneal cells from nonimmunized mice to form anti-sheep cell hemolysins. The reaction was specific because active extracts from mice injected with RRBC failed to convert cells to form antibodies to SRBC.

Although the protein content of the active extracts was not determined and the presence of residual antigen was not excluded, the effect was abolished by pretreatment of the extracts with ribonuclease. Neither RNA extracts from the livers of immunized mice nor the thymus glands of normal mice was active.

There are several reasons to suggest that thymocytes themselves and not bone marrow-derived cells "passing through" the thymus were the cells which responded to antigen. Thymocytes appear not to make antibody, and antibody-forming cells, as detected by the plaque assay method, were not found in the thymus glands of the mice we examined. Gowans and Knight determined

the circulatory route of peripheral blood lymphocytes and concluded that such cells did not pass through the cortex of the gland in significant numbers (14). The rate of incorporation of radioactive uridine into RNA from the thymus increased approximately 16-fold in some fractions on day 3 after immunization, which suggested that a large proportion of the cells of the gland responded. The effect was reversed by the simultaneous injection of anti-mouse thymus serum. RNA-extracts from the thymus glands of immunized mice contained a material which was biologically active, in spite of the fact that significant numbers of antibody-forming cells were not present in the gland. Although it may be that non–antibody-forming cells of bone marrow origin passing through the thymus were the source of this material, the alternative possibility, namely, that the thymocytes themselves yielded the active RNA extracts is more likely. Further, active extracts from the spleens of immunized animals were unable to convert thymocytes from normals to form antibody to SRBC. Thus, if bone marrow derived cells were present, they were in insufficient numbers to be detected by this method or were nonreactive while in the gland. Finally, biologically active RNA extracts from the thymus appeared to be distinct from active extracts from the spleen (Table II). The separation of the extracts, first by sucrose density gradient centrifugation and then by polyacrylamide gel electrophoresis indicated that active extracts from the spleen migrated at a different rate than those from the thymus. While unknown differences, such as aggregation or partial degradation of the molecule(s) may introduce artifactual errors, the results are consistent with distinguishably different active extracts from spleen and thymus.

The role of these newly synthesized RNA's in the immune response is speculative at the moment. The effects of neonatal thymectomy on humoral antibody formation and cell-mediated immunity are well known (15). The requirement for the presence of a functional gland in the recovery of immune responsiveness from the effects of X-ray has been described (16). Animals rendered immunologically tolerant as neonates or immunologically "paralyzed" as adults maintained their specific unresponsiveness for longer periods in the absence of the thymus (17, 18). Thymectomy in the adult resulted in a gradual decrease in the immune potential (19). Davies et al found that thymus-derived cells in lymph nodes divided after the injection of antigen (7, 8).

The infusion of both thymocytes and bone marrow cells into neonatally thymectomized-irradiated animals was required to restore immunocompetence (1, 2). The participation of at least two classes of cells in the immune response implies but by no means proves the existence of a "transfer" substance. Our findings, namely that RNA-extracts from the thymus glands of immunized mice converted nonimmune cells to form antibody, as well as the altered patterns of RNA biosynthesis that occurred after the injection of antigen, may be relevant to this observation.

SUMMARY

The rate of synthesis of RNA in the thymus glands of adult mice increased after immunization with sheep red blood cells (SRBC). The specific activity of some fractions of RNA, separated first by density gradient centrifugation and then by polyacrylamide gel electrophoresis, was 16- fold higher on day 3 after immunization than control mice not injected. RNA synthesis in the thymus was inhibited by rabbit anti-mouse thymus serum, injected along with antigen.

A material was found in RNA extracts from the thymus glands of mice immunized with SRBC which converted a small proportion of either spleen cells or peritoneal cells from nonimmunized mice to form sheep cell hemolysins. Neither extracts from the glands of nonimmunized mice nor the livers of immunized mice were active. Extracts from the thymus glands of mice immunized with rabbit red blood cells (RRBC) were inactive and activity was destroyed by ribonuclease. The residual antigen content was not determined. Biologically active extracts from the thymus had a different electrophoretic mobility from active extracts from the spleen.

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