Proceedings of the National Academy of Sciences
Vol. 67, No. 3, pp. 1299–1306, November 1970

Altered Nucleic Acids of Antibody-Forming Tissues*

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Communicated by Herman N. Eisen, June 12, 1970

Abstract. RNA·DNA hybridization experiments suggest differences between the DNA from the liver and spleen of the same animal (rabbit) and between the RNAs from lymph nodes stimulated by different antigens. A larger proportion of spleen DNA than of liver DNA was complementary to the RNA isolated from antigen-stimulated lymph nodes. No difference between liver and spleen DNA was observed in hybrids formed with RNA from lymph nodes of nonimmune animals. This suggests the presence of unique or redundant genes coding for immunoglobulin polypeptide chains in antibody-forming cells.

RNA·DNA hybrids formed *in vitro* have provided powerful means for relating genes to their RNA products.[1–3] Hybridization of nucleic acids from animal cells has been informative,[8–15] although more complex and controversial than corresponding studies with bacterial or viral nucleic acids.[4–6] Previous results have demonstrated that RNA·DNA annealing can be done with nucleic acids from immunoglobulin-producing tissues.[7,16–20]

In the present study, RNA·DNA hybridization was used to compare DNA from rabbit spleen and liver as well as the rapidly labeled RNAs from rabbit lymph nodes that had been separately stimulated with two antigens that do not cross-react.

Materials and Methods. Preparation of rabbit lymph node cells: These methods are described in detail in a separate publication[21] except that for these studies some rabbits received simultaneous antigenic stimulation with 2,4-dinitrophenyl bovine γ-globulin (Dnp-BγG) and hen egg albumin, each in complete Freund's adjuvant. Such animals were given 0.5 mg of Dnp-BγG divided between the left hind and forepaws and 0.5 mg of egg albumin in the right hind and forepaws. Lymph nodes were excised from axillary and popliteal areas. Separate cell suspensions were prepared in a synthetic medium.[34] Each cell suspension was divided into two approximately equal portions; one served for isolation of unlabeled RNA and the other was incubated with [^{3}H]uridine to provide [^{3}H]RNA.[21]

Isolation of DNA: The procedure of Mach and Vassalli[33] was used to isolate DNA from the quickly-frozen liver and spleen obtained from immunized or nonimmune rabbits. Tissues cut into pieces of approximately 0.5 cm were mixed with an equal volume of phenol for 5 min in a Waring Blendor at 0°C. The homogenate in phenol was then warmed quickly to 60°C and rotated vigorously in a water bath for 10 min. After slow cooling to 25°C, the aqueous phase was separated and 6–7 additional extractions were performed at 60°C, each with fresh phenol. The aqueous phase was then mixed with three volumes of ethanol and the DNA was precipitated at −10°C. After precipitation and washing with ethanol, the DNA was suspended in standard saline citrate (SSC: 0.15 M NaCl–0.015 M sodium citrate) and incubated at 37°C for 30 min

with 10 μg/ml ribonuclease T1 (Calbiochem, Los Angeles, Calif.) plus 10 μg/ml pancreatic RNase (Sigma Chemical Co., St. Louis, Mo., lot no. 638-6820). The pancreatic RNase was previously heated at 80°C for 10 min to inactivate any contaminating DNase. α-Amylase (Worthington Biochemicals, Freehold, N.J.) was then added to a concentration of 10 μg/ml. Digestion at 37°C was continued until the solution was clear; this was followed by a 30-min incubation with 100 μg/ml pronase (Calbiochem). The DNA solution was extracted three times with chloroform–isoamyl alcohol (24:1 by volume). Two volumes of ethanol was added to the aqueous phase and the DNA was spooled out of solution on a glass rod.[22]

Denaturation of DNA: Liver or spleen DNA was diluted to 100–200 μg/ml in 0.1 M Tris·HCl–0.02 M sodium citrate–0.001 M EDTA. The solution was adjusted to 0.5 M NaOH (pH 13), by the addition of 10 M NaOH.[23] After 1 hr at 15–20°C, the solution was chilled, cautiously neutralized to pH 7.0 with 10 M HCl, and dialyzed overnight at 4°C against hybridization buffer.[27]

Labeling and isolation of RNA: Suspensions of rabbit lymph node cells were prepared and incubated *in vitro* as described by Helmreich *et al.*[34] The culture medium was free of nucleotides or nucleosides and supported linear incorporation of [^{3}H]uridine into RNA.[21] After incubation, the cell suspension was chilled to 0°C and [^{3}H]RNA was isolated by repeated extractions with 60°C phenol and chloroform–isoamyl alcohol.[21] Unlabeled liver RNA was isolated by the same technique.

Purification of *E. coli* DNA: *E. coli* DNA (Worthington Biochemicals) was suspended in 0.1 × SSC at a concentration of 500 μg/ml. After several hours of gentle shaking at 23°C, the DNA solution was adjusted to 1 × SSC, and protein was extracted with chloroform–isoamyl alcohol as described by Marmur.[22]

Assay procedures: DNA was determined by the Burton modification[24] of the Dische diphenylamine reaction with the assumption that 51.5% of the bases in DNA of rabbit lymph node cells are purines.[25] Protein concentrations were determined by the method of Lowry *et al.*[26] RNA concentrations were estimated from absorbance measurements at 260 nm and corrected for the presence of DNA, estimated as above (extinctions for 1% solutions were taken as 230 for RNA[27] and 182 for DNA[28]). All RNA preparations *not* subjected to DNase digestion were contaminated with 10–20% DNA.

Hybridization conditions: RNA·DNA hybridization was performed as described by Sly *et al.*[27] New glass test tubes (10 × 75 mm) contained specified amounts of denatured DNA, [uridine-^{3}H]RNA, and 0.06 ml of phenol (saturated with hybridization buffer at 4°C). These were brought to a final volume of 0.6 ml with hybridization buffer, tightly stoppered, and incubated at 60°C. The RNase-resistant hybrids were collected on B6 nitrocellulose filters (Schleicher and Schuell, Keene, N.H.), and the filter-bound radioactivity was measured in a toluene–phosphor scintillation mixture.[29] Duplicate samples of [^{3}H]RNA were coprecipitated with 30 μg of bovine serum albumin in cold 10% trichloroacetic acid for 2 hr, and the precipitates were collected on glass fiber filters for assay of input radioactivity. Hybridization results are expressed as percentage of input radioactivity retained on the nitrocellulose filter.

The capacity of pancreatic RNase to digest nonbase-paired regions of RNA·DNA complexes was tested by incubating two sets of samples containing RNA, DNA, phenol, and buffer. The hybrids in one set were collected on nitrocellulose filters; the second set was incubated with RNase before filtration. Comparison revealed that 20–30% of the filter-bound [^{3}H]RNA was lost after treatment with RNase. This is consistent with the presence of dangling ends and loops of [^{3}H]RNA that were not base-paired with DNA during the annealing reaction.

Hybridization blanks were run in each experiment with *E. coli* DNA and without any added DNA. Competition experiments were done with hybridization mixtures containing a fixed amount of denatured DNA and [^{3}H]RNA ([^{3}H]RNA/DNA ratio 1:6) and various amounts of nonradioactive RNA. Control tubes contained mixtures of RNAs in the same ratio, but no DNA. Tubes for pancreatic RNase activity and apparent binding to *E. coli* DNA were also included for each different RNA/DNA input ratio.

Kennell and Kotoulas have examined and compared the problems of measurement of RNA·DNA hybridization by the three principal methods available—the filter, liquid, and agar-column procedures.[30] Our choice of annealing in solution was based on two considerations: (*a*) Concentration dependence and nonspecific adsorption are less important in the solution method than in the other hybridization techniques.[3] (*b*) The presence of RNase activity contaminating various RNA preparations made it desirable to include an RNase inhibitor in the hybridization reaction mixture. Phenol, which damages nitrocellulose filters, proved to be the most satisfactory inhibitor.

Since we were particularly anxious to use preparations of [^{3}H]RNA that represented the entire cell content of labeled RNA,[21] we sacrificed purity in the interest of high yield, and used RNA preparations known to be contaminated with DNA. DNase was not used in the isolation of RNA since this would have necessitated the temporary removal of the nuclease inhibitors (phenol and detergent) and resulted in the loss of 25–50% of the trichloroacetic acid-precipitable radioactivity (unpublished). Because filtration of the [^{3}H]RNA preparations through nitrocellulose filters did not reduce significantly their content of DNA or of trichloroacetic acid-precipitable radioactivity, it was assumed that the contaminating DNA was not single-stranded and that it did not exist in a stable complex with [^{3}H]RNA. The specificity of the reaction between RNA from rabbit lymph node cells with DNA from the same cells or from other rabbit tissues is shown in Table 1 and Fig. 1 respectively. The inclusion of heterospecific DNA from *E. coli* usually

TABLE 1. *Effects of salt concentration and phenol on the formation of RNase-resistant RNA·DNA hybrids.*

Lymph node cell [^{3}H]RNA (μg)	NaCl concn (M)	Phenol added (ml)	[^{3}H]RNA Bound: To lymph node DNA (cpm)	[^{3}H]RNA Bound: To *E. coli* DNA (cpm)
0.5	0.3	...	209	
0.5	0.3	0.06	365	37
0.5	0.6	...	206	
0.5	0.6	0.06	339	30
0.5	0.9	...	205	
0.5	0.9	0.06	603	39
0.5	1.2	...	190	
0.5	1.2	0.06	496	15
0.5	1.8	...	196	
0.5	1.8	0.06	348	30
2.5	0.9	...	583	
2.5	0.9	0.06	1210	40

RNA from antigen-stimulated rabbit lymph node cells (21,800 cpm/μg) was allowed to react with 5 μg of denatured splenic DNA. In each reaction, the final volume was 0.6 ml; incubation was at 60°C for 18 hr. Each tube was then diluted to 10 ml with 0.01 M Tris·HCl (pH 7.2) and treated with bovine pancreatic RNase as described in the legend to Fig. 1.

resulted in adsorption of even less radioactivity to the nitrocellulose filters than when no DNA was added.

Table 1 and Fig. 1 show that hybridization depends on the ionic strength of the hybridization buffer and on the duration of incubation. The use of 6× SSC (0.9 M NaCl–0.09 M sodium citrate) and incubation for 20–24 hr gave maximum yields. Agreement between duplicate samples in all the hybrid experiments was generally within 10%. Table 1 shows the advantage of having phenol present during the hybridization reaction.[27] Other nuclease inhibitors (such as sodium dodecyl sulfate, 3′:5′-CMP, sodium dextran sulfate, and bentonite) were tested in preliminary experiments and found to be less satisfactory than phenol because of interference with hybrid formation (sodium dodecyl sulfate), physical adsorption of the [^{3}H]RNA (bentonite), or failure to protect [^{3}H]RNA from endogenous nucleases (3′:5′-CMP).

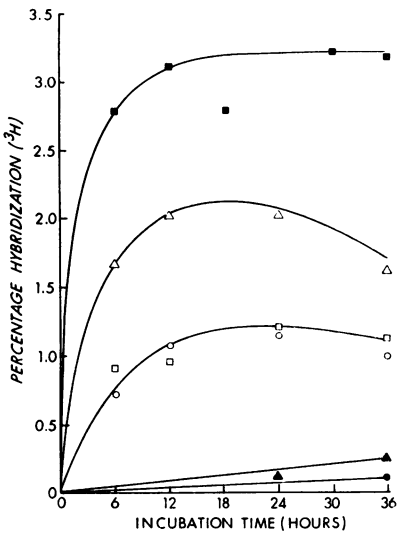

FIG. 1. Time course of hybridization with RNA from rabbit lymph node cells. 5 μg of [³H]RNA was incubated at 60°C with 30 μg of DNA from rabbit liver or spleen in a total volume of 0.6 ml of hybridization buffer containing 0.1 vol of phenol. At each time point, tubes were withdrawn from the water bath and diluted to 10 ml with hybridization buffer. After digestion with 2 μg/ml pancreatic RNase at 37°C for 20 min, the DNA·RNA hybrids were collected on nitrocellulose filters. The radioactivity retained by the filters was corrected by subtraction of the amount bound to filters that contained no DNA. [³H]RNAs were isolated after 1 hr incubation of lymph node cells with [³H]uridine. Hybridization shown is that of [³H]RNA from lymph node cells of immunized rabbits with DNA from nonimmune rabbit spleen (■) or rabbit liver DNA (△); [³H]RNA from lymph node cells of nonimmune rabbits with rabbit spleen DNA (□) or rabbit liver DNA (○); [³H]RNA from lymph node cells of immunized (▲) or nonimmunized (●) rabbits with *E. coli* DNA.

Results. Differences between RNAs from resting and immune cells in hybridization reactions: The following experiments show two marked differences in the RNA populations from immune and nonimmune cells isolated after 1 hr incubation in [³H]uridine. First (Fig. 1), there was greater hybridization of [³H]RNA (% ³H hybridized) derived from antigen-stimulated lymph node cells than with [³H]RNA isolated from nonimmunized animals. Second, spleen DNA gave about 50% more hybridization than liver DNA with [³H]RNA from antigen-stimulated cells. In contrast, [³H]RNA from unstimulated cells failed to distinguish between splenic DNA and liver DNA. Each of these differences was also observed, although to a lesser extent, in similar experiments performed with [³H]RNA obtained from lymph node cells after a longer labeling interval (6 hr) with [³H]uridine.

Fig. 2 shows that rabbit liver RNA competed for only a minor fraction of the [³H]RNA from lymph node cells of nonimmune rabbits. Thus, a substantial fraction of the labeled RNA appears to be unique to lymph node cells. The difference between [³H]RNA from antigenically stimulated and resting lymph

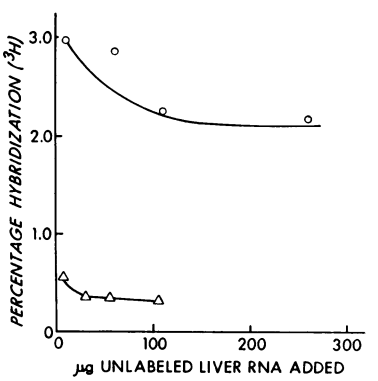

FIG. 2. Hybridization-competition differences between RNA from lymph node cells of immune (○) and nonimmune (△) rabbits. 5 μg of [³H]RNA from lymph node cells (labeled for 1 hr with [³H]uridine) was incubated with 30 μg of rabbit spleen DNA in the presence of various amounts of unlabeled rabbit liver RNA.

node cells (Figs. 1 and 2) must be attributed to differences in the distribution of label or to different kinds of RNA molecules in the two preparations. Unlike the results in Fig. 2, where unlabeled liver RNA was used as competitor, plateaus were not observed when unlabeled lymph node cell RNA was used for competition and there was more competition at lower RNA/DNA ratios (see Fig. 4).

Attempt to saturate DNA sites with lymph node [^{3}H]RNA: An effort was made to saturate the spleen DNA with [^{3}H]RNA from a 6-hr incubation of antigen-stimulated axillary and popliteal lymph node cells with [^{3}H]uridine. An apparent plateau was achieved at 0.185 μg of RNA per μg of DNA in the hybrid (Fig. 3). This value is certainly a considerable overestimate, probably result-

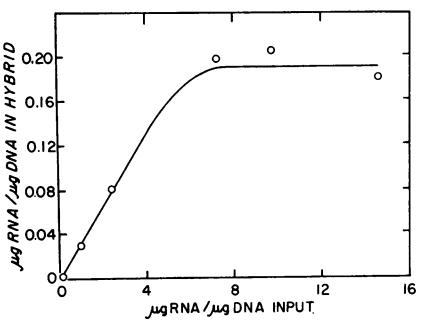

FIG. 3. Approach to saturation of rabbit spleen DNA hybridized with immune lymph node cell RNA. As the RNA/DNA input ratios increased, an apparent plateau was achieved at 0.18 μg of RNA per μg of DNA. Each reaction mixture was prepared as for Fig. 1 except that 1 μg of rabbit spleen DNA was used in each reaction with increasing amounts of [^{3}H]RNA from a 6-hr incubation of antigen-stimulated lymph node cells with [^{3}H]uridine.

ing from a higher specific activity of the hybridized RNA molecules relative to the bulk RNA. Even though the labeling period was relatively long (6 hr), it is probable that the RNA molecules had not reached a uniform specific activity.[21] The apparent plateau in Fig. 3 probably represents diverse mRNA species at these high RNA/DNA input ratios, since the gene sites for tRNA and rRNA are saturated at much lower RNA/DNA inputs. Though the mRNA should be much closer to a steady-state level of labeling, we wish to emphasize that the conversion of "cpm retained" to "μg of RNA hybridized" is an approximation that overestimates the fraction of gene sites saturated.

Hybridization with [^{3}H]RNA from doubly immunized rabbits: Injection of antigen into the foot pad stimulates an immune response mainly in the local lymph nodes draining the injection site.[31,36] This provides opportunities for studying the specificities of RNA molecules induced by different antigens. Fig. 4 provides evidence of hybridization specificity with [^{3}H]RNA from an animal immunized with Dnp-BγG in its left hind and forepaws and hen egg albumin in its right hind and forepaws. Tubes in each series contained rabbit spleen DNA (10 μg), [^{3}H]RNA (1.5 μg) from cells stimulated by either egg albumin or Dnp-BγG, and increasing amounts of unlabeled RNA from homolateral or contralateral lymph nodes. The experiment shown in Fig. 4 is typical of several that consistently indicated preferential competition by RNA from lymph nodes stimulated by the same antigen. Serum samples obtained just before the animal was sacrificed indicated that precipitating antibodies specific for each antigen were present in the circulation. Other experiments showed the importance of selecting animals that were producing antibodies to both antigens in order to

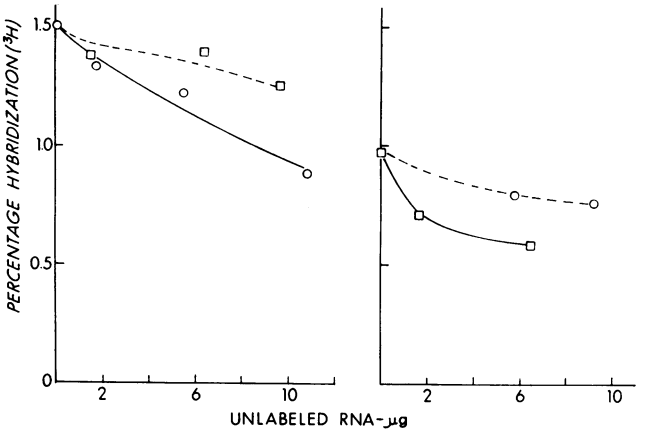

FIG. 4. Hybridization competition with rabbit spleen DNA and [³H]RNA from doubly immunized animals. The hybridization reaction mixtures contained 10 μg of rabbit spleen DNA, 1.5 μg of [³H]RNA from lymph nodes producing antibody to ovalbumin (*left panel*), and unlabeled RNA from the same nodes (○) or from lymph nodes producing antibody to Dnp-BγG (□) in increasing amounts for competition. The right panel shows the corresponding experiments with 1.5 μg of [³H]RNA from lymph nodes producing antibody to Dnp-BγG and the same preparations of DNA and unlabeled RNA.

obtain optimal competition by homologous RNA. Relatively high DNA/RNA input ratios were essential for these measurements. In other experiments, where less DNA was used, competitive differences between RNA from homolateral and contralateral lymph nodes were equivocal.

Discussion. There was more extensive hybridization by RNA from antigen-stimulated cells than from nonimmune cells (Fig. 1). This is consistent with the stimulation of cell proliferation observed in regional lymph nodes after antigenic stimulation and could imply a general increase in total cellular RNA or in certain classes of RNA. However, only a minor difference has been observed in the over-all rate of [³H]uridine incorporation into RNA by antigen-stimulated or unstimulated cells.[21] Thus, it is probable that the differences in percentage of radioactivity hybridized reflect a greater heterogeneity of the labeled RNA species from antigen-stimulated cells. Evidence for heterogeneity was obtained previously in sucrose gradient analyses in which there was a broad zone of labeled RNA centered around the 12–13 S region in preparations obtained from continuously labeled, antigen-stimulated lymph node cells.[21]

The difference in antigen-stimulated [³H]RNA that hybridizes with liver and spleen DNA (Figs. 1 and 2) suggests that there might be unique DNA sequences in spleen cells.[§] A possible explanation for this result is that tissues like spleen, which are rich in antibody-forming cells and their immediate precursors, may contain more immunoglobulin genes than another tissue like liver, which would contain only immunoglobulin genes of germ line cells. Liver also contains lymphoid cells and probably contains antibody-forming cells, but far fewer relative to the total cell mass than spleen. An increased number of immunoglobulin genes in antibody-producing tissues could arise by a mechanism

of specific gene amplification[8] or it might reflect a heterogeneity of immunoglobulin genes arising from mutation or recombination occurring in lymphoid cells.

From Kennell's titrations of the active gene sites on the *E. coli* chromosome,[32] it seems probable that RNA·DNA hybridization could provide a reasonable means of estimating the number of genes being transcribed in the immunoglobulin-producing cell. However, we have not attempted this because constant specific activity of all species of cellular RNA was not achieved.[21] Consequently, it was not possible to assign a single value of specific activity to all classes of RNA (some of which may be highly labeled while others are unlabeled) and the apparent plateau in Fig. 3 could not be used to calculate the number of active genes. It seems possible that such a number could be established if label were continuously provided for several cell-doubling times (e.g., cells in culture) or if the mRNA that codes for immunoglobulin polypeptides could be isolated.[37] It should also be noted that these experiments probably detected only the fastest-reacting RNA and DNA sequences and those in sufficient concentration to be found at equilibrium.[4]

We did not expect that hybridization studies would detect differences in the [^{3}H]RNA from cells responding to different antigens, since there are extensive regions of identity in the amino acid sequences of immunoglobulin polypeptides. It is possible, therefore, that the competition experiments shown in Fig. 4 reflect differences in the cellular kinetics of proliferation in response to different antigens or differences in the synthesis of RNA that has a regulatory role.[35] Nonetheless, it is tempting to believe that the apparent specificity of hybridization reflects differences in mRNA molecules coding for antibodies of different specificities. As much as 60% of the protein secreted by antigen-stimulated lymph node cells may be accounted for as antibodies of a particular specificity;[34] so that despite the cellular heterogeneity in suspensions of rabbit lymph node cells, mRNA specifying a particular product may be abundant.

We express our appreciation to David Kennell for his help in both technical details and interpretation of these experiments.

Abbreviations: SSC, standard saline citrate (0.15 M NaCl–0.015 M sodium citrate); Dnp-BγG, 2,4-dinitrophenyl bovine gammaglobulin.

* This work was supported, in part, by Research Grants AI-03231 and 52742 and Training Grant 5T1-AI-257 from the National Institute of Allergy and Infectious Diseases, and by Contract DA-49-193-MD-2330 with the Research and Development Command, Department of the Army, recommended by the Commission on Immunization of the Armed Forces Epidemiology Board.

† Recipient of a Research Career Program Award (1-K3-AM-38, 620) from the National Institute of Arthritis and Metabolic Diseases.

§ Previous experiments[12] failed to detect such organ-specific differences, perhaps because the RNA used was derived from unstimulated lymphoid tissue; our data indicate likewise that RNA from unstimulated lymph node cells fails to distinguish between liver and spleen DNA during hybridization.

‡ Some of this work was performed in partial fulfillment of the requirements for an M.A. degree awarded by the Committee on Molecular Biology (supported by N.I.H. traineeship, 5 TI GM 00714) of the Graduate School of Arts and Science of Washington University, St. Louis, Missouri.

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