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Symposium on In Vitro Studies of the Immune Response

IV. Role of Nucleic Acids in the Anamnestic Antibody Response[1]

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INTRODUCTION

The antibody response follows the injection of foreign substances, called antigens, into vertebrates, and includes a series of intracellular events which culminate in the synthesis of specific antibody globulins. Antibody synthesis is increased during the anamnestic response to a subsequent injection of antigen several weeks or months after the primary stimulus. The anamnestic response is characterized by extensive mitosis and changes in morphology of the cells of lymphoid organs.

The present study was concerned with the nature of the information which is utilized for antibody synthesis during the anamnestic response. Attention was focused on the role of nucleic acids, because a considerable body of evidence implicates deoxyribonucleic acid (DNA) as a source of information which, with the mediation of ribonucleic acid (RNA) molecules, is transcribed and translated into the amino acid sequence of the specific protein end product (46). Moreover, nucleic acids already had been indirectly implicated in the antibody response. Antibody synthesis is inhibited when animals are X-irradiated (43) or treated with purine and pyrimidine analogues which inhibit mitosis, DNA, and RNA syntheses (34). The inhibition of antibody synthesis by X ray is counteracted by the injection of various nucleic acid derivatives (42). The anamnestic response is characterized by extensive mitosis (28) and increased synthesis of DNA and RNA (5) during the first 2 or 3 days after the injection of antigen before the peak of antibody synthesis is attained. Addition of inhibitors of RNA synthesis to antibody-forming tissues in vitro inhibits antibody production, but this effect may not be specific (7, 40).

Efforts to gain more direct evidence for the roles of DNA and RNA in the antibody response have been hampered by the lack of (i) in vitro systems which rapidly synthesize antibody and can, therefore, quickly reflect sudden changes in this synthetic capacity, and (ii) agents which specifically inhibit DNA and RNA synthesis. The first requirement was met by the development of in vitro systems which employ lymphoid tissue fragments (40) or cells (45) from immunized animals. These systems rapidly incorporate radioactive amino acids into antibody; moreover, this incorporation is a measure of the extent of de novo synthesis of antibody (45). The second requirement seemed to be met by the discovery of the mode of action of the antibiotic, actinomycin D. This antibiotic blocks the synthesis of complementary strands of RNA by binding selectively to the deoxyguanosine residues of the DNA (14), which is required as a template for the function of RNA polymerase (13). Based on these findings, the rationale of the present study was as follows. The antibody synthetic system would be employed in its fully induced state, i.e., during the height of the anamnetsic response 3 days after the last injection of antigen. At this time, lymphoid cells synthesize anti-

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bodies at a maximal rate, and, if explanted into a suitable medium which contains a radioactive amino acid, incorporate this precursor into antibody at a constant rate for 5 to 8 hr (40, 45). Lymphoid fragments from similarly immunized animals under these conditions continue to synthesize antibodies for weeks (40, 26). Various concentrations of actinomycin D would be added to the medium which contained the antibody-synthesizing tissues to determine whether the turning-off of DNA-dependent RNA synthesis affected the synthesis of antibody. The inhibition of antibody synthesis by this antibiotic would suggest that information for the synthesis of a specific antibody was present in DNA, and that this information was then transcribed and translated through the mediation of RNA molecules, as has been suggested for other protein-synthesizing systems (46).

Since the initiation of this study, a number of reports of the effects of actinomycin D on the antibody response have been made. This antibiotic inhibited antibody synthesis partially or completely when it was injected into animals before the antigen was given (12, 19, 39, 47) and when it was added to cultures of tissues from immunized animals (38, 41, 44). In several studies, however, the antibody response seemed somewhat resistant to inhibition by this antibiotic, especially when actinomycin was injected into animals several days after the last injection of antigen (12, 19) or when it was added to cultures prepared several days after the last inoculation of antigen (19, 38, 44). With one exception (38), these studies depended upon the assay of antibody activity in serum or culture medium rather than the demonstration of actual antibody synthesis by the incorporation of a radioactive amino acid into antibody. For this reason, the exact time of cessation of antibody synthesis in vivo or in vitro under the influence of actinomycin D could not be determined. Nor was it determined whether under the conditions of the experiments actinomycin did in fact inhibit DNA and RNA synthesis. In the study by Smiley, Heard, and Ziff (38), the incorporation assay was employed, and the effects of actinomycin on antibody and RNA synthesis were observed. The findings of the present study are quite similar to the findings of these authors, i.e., that, depending upon the dosage of antibiotic, actinomycin may inhibit, stimulate, or be without effect on the antibody response, although it usually inhibits RNA synthesis to varying degrees. The present investigation has revealed additional facts which permit further conclusions and stimulate some speculation about the role of nucleic acids in the antibody response.

MATERIALS AND METHODS

Rabbits were injected twice intravenously or into the hind footpads, with an interval of 21 to 50 days between injections, with one of the following alum-precipitated antigens: 2 to 4 mg of bovine globulin, human serum albumin, keyhole limpet hemocyanin, or 40 Lf units of diphtheria toxoid. Other rabbits were hyperimmunized by the injection of 5 to 10 mg of bovine β-lactoglobulin (23) into the hind footpads every 3 months for 2 to 3 years. The antigens were obtained from the following sources: bovine γ-globulin and human serum albumin, Pentex Laboratories, Kankakee, Ill.; keyhole limpet hemocyanin Pacific Bio-Marine Supply Co., Venice, Calif.; diphtheria toxoid, Lederle Laboratories, Pearl River, N.Y. Three days after the last injection of antigen, spleens were removed from rabbits which were inoculated intravenously, and the popliteal lymph nodes were removed from those which were inoculated into the footpads. Six to eight fragments of tissue, each 1 to 2 mm^3, were fixed to stainless-steel grids with sterile agar and placed in 1 ml of Eagle's medium (8) containing 1 μc of glycine-*1-C*^{14} (15 $\mu c/\mu mole$) in a 13 $\times$ 100-mm tube. Alternatively, cells were teased out of the tissues and 10^6 to 8 $\times$ 10^6 nucleated cells were placed into the medium which contained the labeled amino acid. As a precursor of RNA, 0.25 μc of uridine-*2-C*^{14} (New England Nuclear Corp., Boston, Mass.) with a specific activity of 0.2 $\mu c/\mu mole$ was placed in the medium. As a precursor of DNA, 0.25 μc of thymidine-2-C^{14} (New England Nuclear Corp., Boston, Mass.) with a specific activity of 1.0 $\mu c/\mu mole$ was used. The tubes were then incubated in a roller drum at 37 C.

Extracellular labeled antibody was assayed in the medium after removal of the cells by centrifugation, or removal of the fragments by pulling the grids out of the tubes. Nonspecific radioactivity was partially removed from the medium by dialysis against several liters of saline for 24 hr at 5 C and by two or more coprecipitations with an antigen-antibody precipitate which did not cross-react with the antibody under study. The specific labeled antibody was then coprecipitated from the supernatant fraction of the last nonspecfic precipitate by repeated additions of unlabeled homologous antigen and antibody. For counting, the precipitates were collected on a filter membrane and counted in a low-background Nuclear-Chicago flow counter. Radioactivity of intracellular antibody was determined by the application of the same procedure to a centrifugally clarified homogenate of the cell suspension after incubation. Radioactivity of cellular RNA and DNA was determined by Kahan's method (20),

with samples of about 10^6 well-washed lymphoid cells.

RESULTS

Effects of Actinomycin on Synthesis of Antibody, Nonantibody Proteins, DNA, and RNA

A 1- to 10-μg/ml amount of actinomycin caused prompt cessation of RNA and DNA synthesis, followed within 1 to 3 hr by complete or partial inhibition of synthesis of antibody to human serum albumin (Fig. 1). At these levels, the compound also inhibited the synthesis of nonantibody proteins, including α-, β-, and γ-globulins. A 0.1- to 0.5-μg/ml amount of actinomycin inhibited RNA synthesis, but only partially inhibited the synthesis of antibody to hemocyanin and the synthesis of nonantibody proteins (Fig. 2). The synthesis of DNA was only partially inhibited at these levels. Figure 3 illustrates the results of another experiment with hemocyanin in which the dissociation of the effects of actinomycin on RNA and on antibody synthesis is particularly clear. Similar results were observed in numerous experiments upon the addition of this antibiotic to lymph node and spleen cells and fragments which were synthesizing antibodies to bovine γ-globulin, bovine β-1 actoglobulin, and diphtheria toxoid.

Occasionally, upon addition of lower doses of actinomycin, stimulation of antibody synthesis was observed (Table 1). Stimulation usually was transitory, and eventually synthesis of antibody approached control values or was somewhat inhibited (Table 1). At these levels of antibiotic, some inhibition of RNA synthesis occurred (Table 1), but it was never complete.

Since previous (45) and recent experience indicated that isolated lymphoid cells lost the capacity to synthesize antibody more rapidly than did lymphoid fragments, the long-term effects of actinomycin were investigated with tissue fragments. Table 2 shows that some antibody synthesis continued for at least 24 hr when lymph node fragments were treated with 1 and 5 μg/ml of antibiotic, which inhibited RNA and DNA synthesis completely.

Brief exposure of cells or fragments to 1 μg/ml of actinomycin for 1 hr resulted in inhibiton of antibody synthesis. The effects of this compound resulted from binding to cellular components, since addition of deoxyguanosine to the medium with actinomycin relieved the inhibition (Table 3).

The reduction in extracellular labeled antibody in actinomycin-treated cultures might have been caused by an inhibition of secretion of antibody which usually is rapidly secreted into the medium (18). However, it was found that, in the presence of this antibiotic, the cells liberated more labeled antibody than the untreated cells (Table 4).

Cells which were prepared from lymph nodes of immunized rabbits 6 days after the last injection of antigen synthesized much less antibody in vitro than cells from nodes which were removed on day 3. Nevertheless, the synthesis of antibody by these cells was also only partially inhibited by concentrations of actinomycin which were adequate to inhibit RNA and DNA synthesis completely.

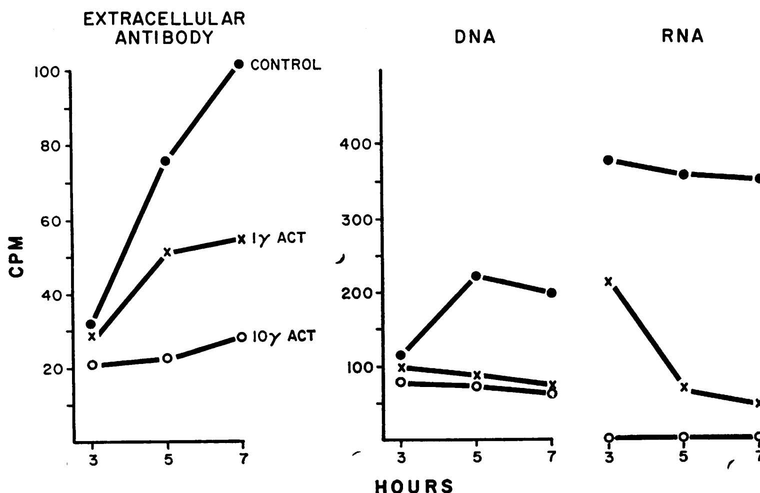

FIG. 1. *Effects of actinomycin D on the synthesis of DNA, RNA, and antibody to human serum albumin by lymph node cells of rabbits immunized with this antigen. The count/min of extracellular antibody represents the sum of the counts associated with each of the specific HSA-antiHSA precipitates.*

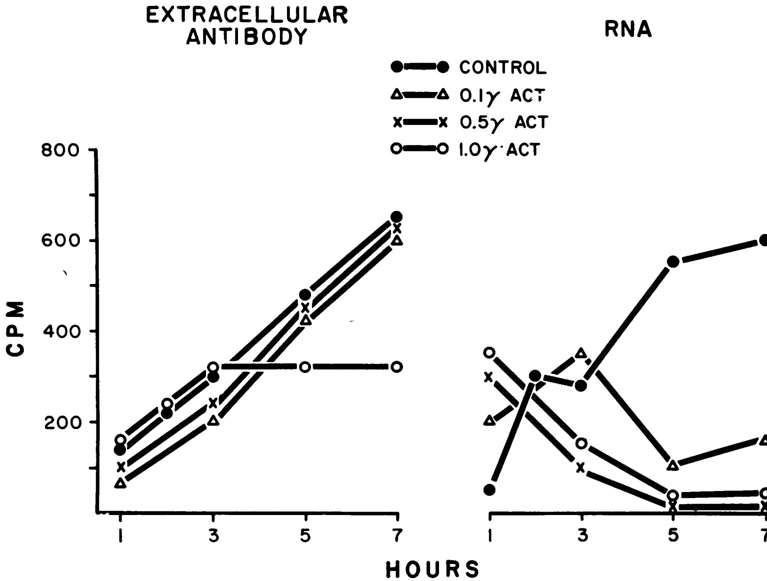

FIG. 2. *Effects of actinomycin D on the synthesis of antibody to hemocyanin, nonantibody protein, and RNA by lymph node cells from rabbits immunized with this antigen. Extracellular protein refers to the nonantibody protein, mainly globulins, which were precipitated from the medium by 50% $(NH_4)_2SO_4$ after the removal of the antibody by coprecipitation with specific immune precipitates.*

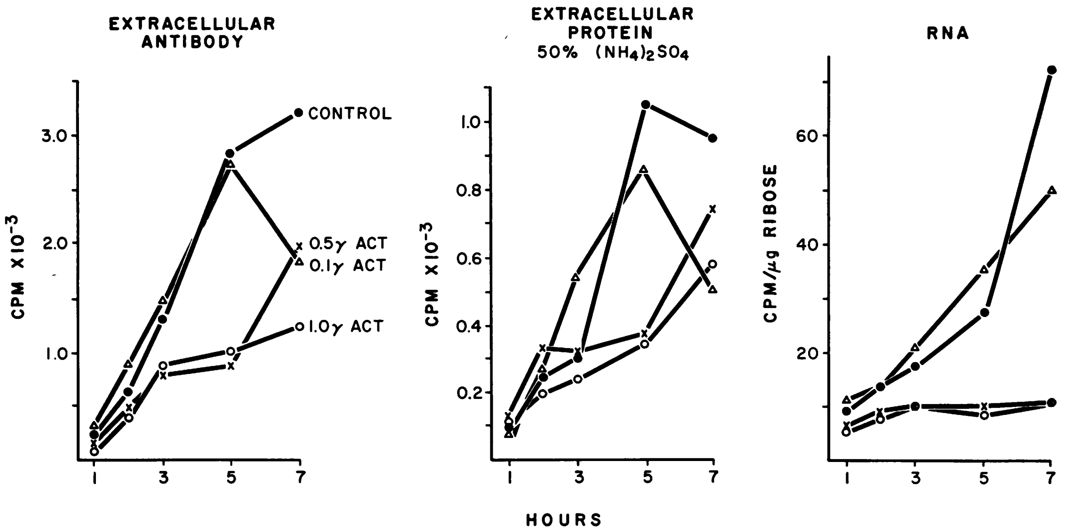

FIG. 3. *Effects of actinomycin D on the synthesis of RNA and antibody to keyhole limpet hemocyanin by lymph node cells of rabbits immunized with this antigen.*

Effects of Actinomycin on Catabolism and Leakage of RNA, DNA, and Antibody

The inhibitory effects of actinomycin on antibody synthesis could be attributed to the inhibition of DNA and RNA synthesis which was shown in Fig. 1, 2, and 3. However, inhibition of antibody synthesis might have been caused by increased catabolism of DNA, RNA, or antibody. Experiments to test these alternate explanations revealed that actinomycin did promote the breakdown of cellular RNA (Table 5). Less effect on the catabolism of DNA was observed. Lower concentrations of actinomycin caused minimal effects on the catabolism of RNA, but even with 0.1 μg/ml, some increase in the breakdown of cellular RNA could sometimes be observed.

TABLE 1. *Stimulation of synthesis of antibodies to hemocyanin upon addition of actinomycin to lymph node cells in vitro*

Hr incubated	Actinomycin D	Total antibody*	RNA†
	μg/ml	*count/min*	*count/min*
3	—	239	465
5	-	567	---
7	—	1,185	1,084
3	0.01	398	419
5	0.01	672	610
7	0.01	806	535

* Extracellular plus intracellular antibody.
† Total cellular RNA.

TABLE 2. *Effect of actinomycin D on synthesis of antibodies to hemocyanin by lymph node fragments in vitro**

Hr incubated	Count/min in extracellular antibody		
	Control	1 μg/ml	5 μg/ml
3	129	200	122
7	162	234	156
24	820	467	285

* The control was incubated in the absence of drug; the experimental in the presence of the indicated amounts of drug.

TABLE 3. *Relief of inhibition of synthesis of antibodies to hemocyanin by actinomycin D upon addition of deoxyguanosine to lymph node cells*

Incubation mixture	Count/min in extracellular antibody*
Control	160
10 μg of actinomycin D	100
340 μg of deoxyguanosine	152
10 μg of actinomycin + 340 μg of deoxyguanosine	125

* After 3 hr of incubation.

The effect of actinomycin on the catabolism of antibody was observed by following the rate of decline of labeled extracellular and intracellular antibody during the course of incubation of lymphoid tissues and by adding labeled antibody to such cultures in the presence and absence of actinomycin. Although only a few experiments were done, there was no indication of enhanced breakdown of antibody in the presence of actinomycin.

TABLE 4. *Radioactivity of extracellular and intracellular antibody to bovine β-lactoglobulin synthesized in presence and absence of actinomycin D*

Hr incubated	Antibody	Count/min in antibody		
		Control	0.5 μg[a]	1.0 μg[a]
3	Extra[b]	76	88	74
	Intra[c]	103	91	119
	Total[d]	179	179	193
5	Extra	151	143	128
	Intra	193	96	108
	Total	344	239	236
7	Extra	151	167	149
	Intra	120	112	97
	Total	271	279	246

[a] Actinomycin D.
[b] Extracellular.
[c] Intracellular.
[d] Extracellular + intracellular.

TABLE 5. *Increased destruction of cellular RNA upon incubation of lymphoid cells with actinomycin*

Incubation mixture	Count/min in RNA after 1-hr pulse*	Count/min in RNA after 2-hr chase†
Control cells	680	320
10 μg of cells	666	129

* One hour after exposure of cells to uridine-2-C^{14} at 37 C.
† Cells were washed after the 1-hr pulse, resuspended in 200 μg of unlabeled uridine, and incubated in the absence of actinomycin (control cells) or in the presence of 10 μg of actinomycin (10 μg of cells) for an additional 2 hr at 37 C.

Effects of Actinomycin D on Cell Viability

Exposure of lymphoid cells to concentrations of actinomycin up to 10 μg/ml for up to 8 hr did not result in any clear-cut morphological effect as observed by phase-contrast microscopy. By exposure to this drug, the number of cells was not reduced, compared with untreated control cells. Finally, it was observed that the numbers of untreated and actinomycin-treated cells (up to 10 μg/ml) which took up trypan blue during 8 hr of incubation at 37 C were not significantly different.

Species of Antibody Synthesized in Presence and Absence of Actinomycin

Sucrose density-gradient ultracentrifugation (9) was employed to separate antibodies of dif-

TABLE 6. *Species of antibody to hemocyanin synthesized by lymphoid tissues in vitro in presence and absence of actinomycin D*

Actinomycin	Sucrose fraction	Count/min
—	1-3 top	22.0
	4-6 7*S**	68.5
	7-8	0
	9-11 19*S*†	0
25 μg	1-3	18.0
	4-6	22.0
	7-8	0
	9-11	0

* Rich in 7*S* proteins.
† Rich in 19*S* proteins.

ferent molecular weights from the media after culture of lymphoid cells from immunized animals. The incorporation assay was then applied to these fractions. It is apparent from Table 6 that mainly, if not exclusively, 7*S* antibody was synthesized in these cultures and that it was the synthesis of this species of antibody that was inhibited.

DISCUSSION

Rabbit lymphoid cells synthesized antibodies to hemocyanin and human serum albumin in vitro for at least 1 day in the presence of enough actinomycin to inhibit RNA and DNA synthesis completely. The response of rabbit tissues to diphtheria toxoid, bovine γ-globulin, and β-lactoglobulin and of human lymphoid tissues to tetanus toxoid (17) was similarly affected by this agent. Similar data were obtained in another laboratory with rabbit lymph node tissues which were synthesizing antibodies to bovine serum albumin (38). The use of the incorporation assay in these two studies excluded the possibility that the continued appearance of antibody in the actinomycin-treated cultures resulted from the release of intracellularly stored antibody. These results indicate that antibody synthesis is somewhat independent of continued nucleic acid synthesis. The data are consistent with the findings that the peak of DNA synthesis precedes the peak of antibody synthesis in populations of lymphoid cells (28), and that most cells which contain immunoglobulins are not synthesizing DNA (3). The results also agree with the observations that 5-bromodeoxyuridine, which is incorporated into DNA and is toxic for cultured mammalian cells (25), does not completely inhibit the anamnestic response when added to tissues several days after antigenic stimulation (6, 30). Finally, these data are in line with the findings of mammalian systems in which protein synthesis continued for many hours or days in the absence of RNA synthesis (4, 32, 37)

Antibody synthesis presumably continues in the absence of DNA and RNA synthesis, because one or more of the cellular RNA fractions contains pertinent and stable information. Whether the same information is utilized for the continuation of antibody synthesis in the absence of antigen or for the maintenance of "immunological memory," i.e., the capacity to manifest an anamnestic response, is not known. It is also not known whether this information remains in a cell during the lifetime or whether it can be transferred to another cell in which it can then function. Whether or how this information is regulated in the course of the antibody response by factors such as level of antigen and cellular differentiation remains to be determined.

A 1- to 10-μg/ml amount of actinomycin, which partially or completely inhibited antibody and nonantibody protein synthesis as well as DNA synthesis, completely inhibited RNA synthesis and promoted the breakdown and leakage of cellular RNA in lymphoid cultures. The variety of effects precludes the simple explanation that actinomycin inhibits antibody synthesis by interfering with the synthesis of the requisite RNA templates. Addition of 0.01 to 0.5 μg/ml of actinomycin to lymphoid cultures occasionally inhibited antibody synthesis with minimal effects on RNA. The latter observations provide the strongest evidence available at present for the roles of DNA and short-lived RNA in the anamnestic antibody response. Such roles would be consistent with the findings of amino acid differences between antibodies of different specificities (21) and with data for the colinearity of gene structure and the amino acid sequence of its protein end product (48). Recently, evidence has been adduced for in vitro synthesis of altered antibodies with changed specificity upon addition of streptomycin to lymphoid cultures (22). Presumably, the streptomycin introduced amino acid changes into the antibody molecule by altering the translation process whereby the messenager RNA is decoded at the ribosomal level.

Antibody synthesis was reduced to about 50% of control values 4 to 5 hr after the addition of 1 to 10 μg/ml of actinomycin, which is adequate to cause immediate cessation of all RNA synthesis in lymphoid cell cultures (Fig. 1, 2, and 3). However, considerable antibody synthesis (one-third to one-half of control values) often continued for at least 1 day, the longest period of observation, under these circumstances (Table 2). These data suggest that there are at least two classes of RNA

with information for antibody synthesis with half-lives of 4 to 5 hr and at least 24 hr, respectively. Recently, evidence has been obtained for the presence of RNA of short and long half-lives in other mammalian protein-synthesizing systems (15, 16, 31, 36).

Low concentrations of actinomycin occasionally stimulated antibody synthesis, a finding also noted in another study (38). The mechanism of this stimulation is not known, but it often was accompanied or preceded by stimulation of cellular RNA synthesis. Recently, the stimulatory effect of actinomycin on the induction of protein synthesis in other systems has been reported (11, 33).

The various effects of actinomycin on the in vitro anamnestic response can be explained in a number of ways. The possibility that this drug inhibited the synthesis of one species of antibody, but permitted the synthesis of another species, was first considered and rejected because it was found that the cultures synthesized only 7*S* globulin antibodies (Table 6). However, recent studies revealed considerable heterogeneity among the 7*S* immunoglobulin molecules in several species (10), and it is possible that differential inhibition of the synthesis of different species of 7*S* globulin molecules occurs. A more likely explanation has been proposed: that lymphoid cells contain labile and stable RNA molecules for the synthesis of antibodies. Either single cells possess both types of RNA, or individual cells contain one or another of these types for antibody synthesis (15, 16, 31, 36). By analogy with studies of cellular differentiation in other systems (27, 35), the two types of cells may be undergoing induction or have already been induced. The cells which are being induced would be expected to utilize DNA for the synthesis of template RNA. This RNA would serve to synthesize proteins which are required for mitosis, cellular differentiation, and antibody synthesis. The capacity of these cells to divide, differentiate, and synthesize antibody would be inhibited by actinomycin. The cells which are already induced for these functions would not synthesize RNA, but would continue to synthesize antibody in the presence of actinomycin. Some support for this conception of cellular heterogeneity in antibody production is provided by the demonstration in the present study (17) and in a study from another laboratory (2) of a wide diversity of morphological types of cells which contain antibody. Further support is derived from finding that blast cells in rat lymph nodes turn over RNA extensively, whereas mature plasma cells do not (29). Induction for one or more function(s) may occur on day 3 and on day 6 of the anamnestic response, initially for mitosis and cell differentiation, and later, perhaps, exclusively for antibody synthesis. Moreover, all of these systems seem dependent to some extent upon the continued synthesis of RNA with a half-life of about 4 to 5 hr. Previous studies with 5-bromodeoxyuridine (6, 30), actinomycin (19, 44), and chloramphenicol (1) suggest that little stable RNA for the anamnestic antibody response is available during the first 3 days after antigenic stimulation. The antibody response seems more susceptible to inhibition by these agents at this time. Studies of this period are in progress.

During the preparation of this manuscript, a paper by Lazda and Starr has appeared (24). They determined the effects of actinomycin on the synthesis of RNA and of antibodies to dinitrophenyl-bovine γ-globulin by rabbit spleen fragments. They found that fragments obtained at the height of the anamnestic response continued to synthesize antibodies for at least 18 hr after preincubation with 1 μg/ml of antibiotic. Total cellular RNA synthesis was inhibited about 76% and was limited to nucleotide incorporation into soluble RNA. They considered the results consistent with the hypothesis of a relatively stable messenger RNA in antibody synthesis.

Although the available data suggest that short-lived and long-lived RNA are involved in the anamnestic antibody response, much more information is needed. There is no evident correlation between the levels of RNA synthesis and antibody synthesis in lymphoid organs. This is not surprising, since only a small percentage of the cells are making antibody, only a small percentage of the antibody-forming cells are making RNA, and the same cells are not necessarily performing both syntheses. More detailed analyses of the various cellular RNA fractions of normal and immunized animals in the presence and absence of agents like actinomycin are needed. Determinations of the sedimentation constants, base composition, and stimulatory effects in in vitro antibody-synthesizing systems of the rapidly labeled RNA may reveal whether a messengerlike RNA functions in the antibody response.

The available data can be interpreted according to the recently developed model of the transcription of genomic information into RNA and the subsequent translation of this information on the ribosomes into the amino acid sequence of specific proteins (46). However, it is clear that more detailed analysis is required to relate this model to the various steps of the antibody response. It is not at all clear, for instance, how the antigen functions to induce the antibody-forming system initially.

SUMMARY

Different concentrations of actinomycin were added to cultures of lymph node or spleen cells or fragments which were prepared at the height of the anamnestic antibody response to proteins. A 5- to 10-μg/ml amount of actinomycin completely inhibited the synthesis of RNA, DNA antibody, and nonantibody proteins. In the presence of 0.1 to 1.0 μg/ml of this antibiotic RNA and DNA synthesis were inhibited completely, but considerable antibody synthesis continued for at least 1 day. A 0.01- to 0.05-μg/ml amount of drug inhibited RNA and DNA synthesis partially and occasionally stimulated or inhibited antibody synthesis partially. It is postulated that stable messenger RNA mediated the synthesis of antibody in the absence of DNA and RNA synthesis in lymphoid cells. Short-lived messenger RNA may be involved with antibody synthesis which is inhibited by actinomycin. However, the multiple effects of actinomycin on processes other than RNA synthesis, such as the inhibition of DNA synthesis and the promotion of breakdown of cellular RNA and leakage of RNA from the cell, raise the possibility that actinomycin inhibits antibody synthesis by nonselectively damaging the lymphoid cells. More detailed analysis of the system is required to make clear the subcellular mechanisms of the antibody response.

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