ON THE RNA SYNTHESIS IN NUCLEI FROM IMMUNIZED RABBIT LYMPH NODE CELLS

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1. Introduction

Most of the published work about the transformation of lymphoid cells from the resting state towards an activated one have been performed using non-specific mitogens, such as phytohemagglutinin (PHA). On the contrary, a few experiments have been carried out using antigenic stimulation. However, it is reasonable to think that the two processes may be substantially different: as matter of fact, it has been recently found that differences exist in the pattern of DNA replication [1].

As a step toward understanding some of the molecular mechanisms involved in the response to a specific antigenic stimulation, we present here experiments about the conditions of transcription by lymphocyte nuclei from immunized animals.

2. Materials and methods

Adult Bouscat rabbits received in each of their 4 footpads 1 ml of an emulsion composed of equal parts of soluble ovalbumin in saline (2 mg/ml) and complete Freund's adjuvant (Difco CFA). 4 weeks later, the rabbits were given a first intramuscular injection of 0.5 mg ovalbumin, then after 24 hr, a second one of 2 mg. 6 hr later an intramuscular injection of 20 mg promethazine (Specia Phenergan) was given, followed 30 min later by an intravenous injection of 2 mg ovalbumin in 1 ml PBS. The controls were rabbits receiving CFA without ovalbumin according to the same schedule of injections.

The nuclei from lymph node cells were prepared by a modification of the method described by Takakusu et al. [2]. Briefly, the procedure involves cautious homogenization in a medium composed of 0.25 M sucrose, 0.0025 M $MgCl_2$, 0.025 M Tris-HCl pH 7.1, filtration through gauze, successive cycles of washing and centrifugation for 15 min at 300 *g*, then treatment by 0.25% sodium deoxycholate and 0.5% Nonidet P 40. For studying the RNA synthesis in these preparations, the reaction mixture was as follows: 140 μM NH_4SO_4 (previously adjusted to pH 8.1); 50 μM Tris-HCl pH 8.1; 2 μM $MnCl_2$; 7 μM 2-mercaptoethanol; 3 μM NaF; 0.2 μM ATP; 0.2 μM CTP; 0.2 μM GTP; 0.2 μM UTP. Each sample contained 0.1–0.3 μCi of each nucleotide.

Nuclei were added in 0.1 ml of suspension containing about 200 μg DNA. The final vol was 0.5 ml. Incubation was done at 37° for 10 min and it was shown that under these conditions the amount of synthesized RNA was a linear function both of the incubation time and the number of added nuclei (until 300 μg DNA). DNA was estimated by the diphenylamine reaction [3] after perchloric acid extraction of soluble nucleotides and delipidation.

3. Results

3.1. *RNA synthesis by lymphocyte nuclei*

In the first series of experiments, the incorporation of labelled nucleotides by lymphocyte nuclei was measured either from immunized or non-immunized rabbits; this measurement being done using a double

Table 1
Activity of RNA polymerase of lymphocyte nuclei from rabbits immunized with ovalbumin in complete Freund's adjuvant (CFA).

Animals	Days after first antigen injection	AMP + UMP Incorporation*	AMP + UMP Increase in the incorporation**	GMP + CMP Incorporation*	GMP + CMP Increase in the incorporation**	$\frac{A+U}{G+C}$
Control (CFA)	21	1606	1.47	2100	1.46	0.76
Immunized		2365		3080		0.77
Control (CFA)	24	404	2.63	533	2.44	0.76
Immunized		1063		1299		0.82
Control (CFA)	35	533	1.93	690	1.80	0.77
Immunized		1026		1238		0.83
Control (CFA)	35	2291	2.98	3088	2.03	0.74
Immunized		6820		6262		1.09

Nuclei were prepared from rabbits of the same litter; the controls were normal animals receiving only complete Freund's adjuvant (CFA); the immunized animals received ovalbumin in CFA. Nuclear suspensions were added to the reaction medium and RNA polymerase activity measured after 10 min incubation, as described in "Material and Methods".

* in pmoles/10 min/mg of DNA.
** Immunized/control.

labelling procedure: when ATP and UTP were ^{3}H-labelled, GTP and UTP were ^{14}C-labelled and vice-versa. As shown in table 1, quantitative as well as qualitative differences were obtained. As concerns the quantitative ones, the nuclei from antigen-stimulated animals appear to show a level of incorporation clearly greater than the nuclei from controls.

As concerns the qualitative features, the enhancement of incorporation is proportionably greater for the A + U incorporation than for the G + C one; from that, it follows that the ratio (A + U)/(G + C) increases in the nuclei of immunized animals. These differences are significant at a confidence interval of 80%.

3.2. *Residual RNase activity*

Although these experiments were carried out in a fluoride-containing medium, which inhibits a number of RNases, it was of interest to look for the presence in the preparations of some residual RNase able to degrade the neo-synthesized RNA. The procedure we used was as follows: two series of incubations were carried out simultaneously, the first one (series 1) was a 10 min incubation for the determination of RNA polymerase activity, the second (series 2) was a 10 min incubation followed by another 10 min incubation in the presence of 10 μg actinomycin D. If some degradation had taken place, it could be expected that the radioactivity in series 2 would be lower than that in series 1. However, as shown in table 2, negligible changes were observed, whether or not the animals were immunized. Such

Table 2
Residual RNase activity in lymphocyte nuclei as assayed by spontaneous degradation of *in vitro* transcribed RNA.

Procedure of immunization	*In vitro* synthesized RNA (sum of the 4 nucleotides incorporated in pmoles/mg of DNA):		Residual RNAse activity (obtained by subtracting the values of series 2 from those of series 1)
	At the end of the first period of incubation (10 min) (Series 1)	At the end of the second period of incubation (10 min plus 10 min with actinomycin added) (Series 2)	
Control (CFA)	1853	1750	103
Immunization with ovalbumin in CFA	2475	2360	115
Difference	622	610	12

Two samples of reaction mixture were incubated as usual. After 10 min, the incorporation was stopped in the first sample with trichloroacetic acid and the incorporation measured according to the usual procedure; in the parallel sample, the incorporation was stopped by adding 10 μg actinomycin D, and incubation prolonged 10 more min. The radioactivity then was determined. The difference between the amounts of incorporated nucleotides at 10 min (column 1) and 20 min (column 2) represents the RNA degradation occurring during 10 min at 37°.

Table 3
Class A and B RNA polymerase activity in lymphocyte nuclei as assayed with α-amanitin.

Procedure of immunization	Total RNA polymerase activity (pmoles nucleotides/10 min/mg of DNA)	RNA polymerase activity sensitive to α-amanitin (class B) (pmoles nucleotides/10 min/mg of DNA)	RNA polymerase activity insensitive to α-amanitin (class A) (pmoles nucleotides/10 min/mg of DNA)	Activity (% of total activity) related to enzymes of	
				class A	class B
Control (CFA)	1853	1599	254	13.7	86.3
Immunization with ovalbumin in CFA	2475	2114	361	14.6	85.4
Difference	622	515	107		

At time zero 1 μg of α-amanitin was added, then the nucleotide incorporation (sum of the 4 incorporated nucleotides) was determined both in the α-amanitin inhibited samples and in control samples without the drug. The α-amanitin insensitive activity is reportedly related to the class A enzymes. By subtracting the latter from the total activity, activity of the class B enzymes was obtained.

a result renders highly improbable that the difference of incorporation during the first 10 min was due to the absence (or inactivity) in immunized nuclei of a nuclear RNase which, by contrast, would be present in non-immunized nuclei.

These data allow us to ascertain that the quantitative difference of incorporation between nuclei from immunized and non-immunized animals represents the actual effect of the specific immunization.

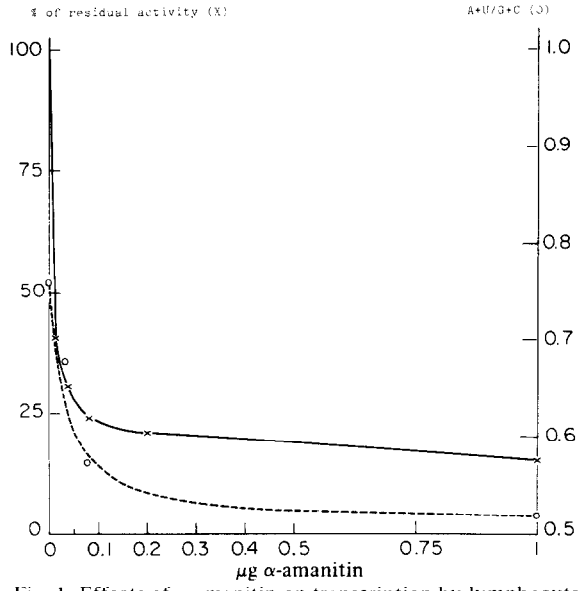

Fig. 1. Effects of α-amanitin on transcription by lymphocyte nuclei. The α-amanitin was added at time zero of incubation. The experiment was then carried out as described in the text.

3.3. *RNA polymerases sensitive or not sensitive to α-amanitin*

Using α-amanitin, it is possible to obtain some information about the qualitative difference reported. Indeed, according to the nomenclature of RNA polymerases recently proposed [4], the class A includes at least 3 enzymes insensitive to the drug, 2 of which, localized in the nucleolus, are mainly involved in the synthesis of a G + C ribosomal-like RNA; the class B includes 2 enzymes sensitive to α-amanitin, generally dispersed through the nucleoplasm and involved in the synthesis of a more DNA-like RNA. Thus, we observed (fig. 1) that in nuclei both from immunized and from the control rabbits, up to 85% of RNA polymerase activity was inhibited by adding 0.2 μg α-amanitin (75% inhibition with 0.1 μg of drug); moreover the greater the inhibition, the lower was the ratio (A + U)/(G + C), which reached 0.5 at the plateau of inhibition. Finally, as shown in table 3, the respective contributions of both the enzymes sensitive (class B) or not sensitive (class A) to α-amanitin can be determined. It appears that in absolute values of radioactivity, the enhanced fraction of transcription due to immunization mainly involves enzymes of class B. But it can be seen clearly that the percentage of the enzymes of either class is the same, whether or not the animals were immunized.

4. Discussion

The results reported here present various aspects. First, they show that the amount of RNA synthesized by the nuclei from lymph node cells of ovalbumin-immunized rabbits is greater than that of the nuclei from non-immunized ones. This increase was consistently observed in all of the present experiments, and the same result was recently obtained by comparing lymphocyte nuclei from rabbits immunized with heat aggregated ovalbumin without Freund's adjuvant to nuclei from normal rabbits [5]: thus 2 procedures of immunization, known to provoke distinctive patterns of morphological and cell repartition changes in the draining lymph nodes involve identical enhancement of RNA synthesis. Variations in the extent of this enhancement are very likely related to the well known differences in the extent of the immune responses between genetically heterogenous animals. However, it is interesting to notice that the proportion of lymphocytes implicated in the process represents a relatively small fraction of the draining node cells. Thus, the values of enhancement which we observed are derived from the contribution of a lower number of nuclei than the number used for the experiments. Relevant to this is the observation that despite the high proportion of transformed lymphocytes following PHA stimulation, only a doubling of RNA polymerase activity is generated [6].

That this enhancement represents an actual increase in the level of transcription is supported by the observation that residual RNase activity was very low both in the immunized and control nuclei. Using a method of double labelling, we have been able to establish that the (A + U)/(G + C) ratio increased after immunization. This characteristic modification was mainly concerned with the activity of RNA polymerases of class B. The fact that the percentage of inhibition by α-amanitin was the same (85%) with immunized or non-immunized animals means that the activity dependent on the class A enzymes increases parallel to that of the class B ones.

Such a situation which implicates ordered changes in the base composition of the DNA-like RNA may be compared with that in embryonic development, where the progressive changes in the nature of the RNA synthesized is not accompanied by changes in the ratio of class A to class B enzymes [7]. Quantitative, as well as qualitative, changes reported here could be ascribed either to enzymes themselves and their associated regulatory factors or to modifications in the template properties of chromatin. However, work in progress in this laboratory [8] showing an identical level of transcription by a purified bacterial RNA polymerase, whether or not the chromatin was prepared from immunized lymphocytes, supports the idea that modifications in template properties of nuclei are not the prominent cause of the quantitative changes provoked by immunization.

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