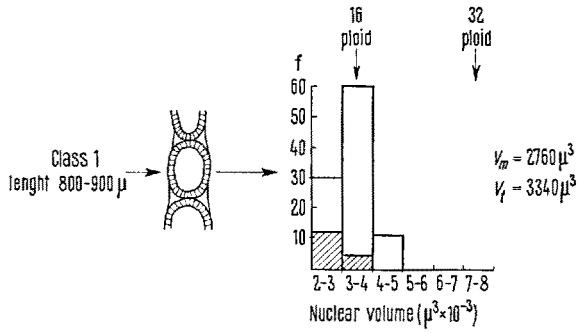

Fig. 1.

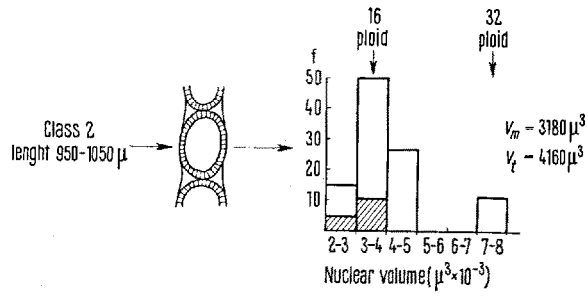

Fig. 2.

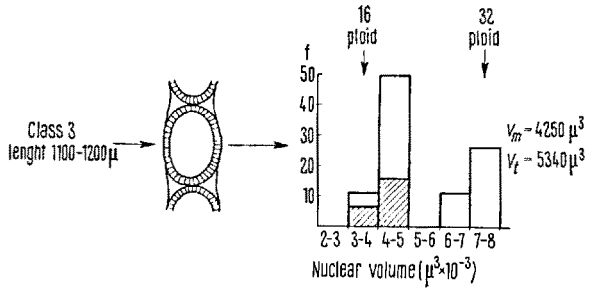

Fig. 3.

▨ labelled nuclei; □ unlabelled nuclei; v_m, average of the nuclear volumes of the labelled cells; v_t, general average of the nuclear volumes, f, frequency (%).

value '470 μ^3' for the diploid volume. The average of the nuclear volumes of the labelled cells and the general average of their class, which is always higher, are shown class by class in the histograms. It can be seen from the histograms that tritiated thymidine is preferentially incorporated into those follicular cells which, in each ovocyte, are of smaller volume than the others. This is so of all 3 classes of ovocytes examined.

Conclusions. Follicular cells have nuclei which increase their size gradually with the growth of the ovocyte. Differences in size exist between the nuclear volumes of the follicular cells of a singly ovocyte. The experiments with tritiated thymidine showed that in every class of follicular cells only the minus variants show incorporation. After incorporation there is a gradual increase in the size of the nuclei. Periods of rapid nuclear growth probably correspond to the empty spaces of the histograms of Figures 2 and 3.

Once growth has subsided a new wave of DNA synthesis starts in the nuclei as can be seen from the histograms of the follicular cells of the larger ovocytes.

Riassunto. Le cellule follicolari di un medesimo follicolo hanno nuclei che diversificano notevolmente fra loro per il volume. Ciò è ben osservabile nelle sezioni trasversali dove i nuclei, di forma cilindrica, appaiono circolari. La timidina tritiata viene incorporata preferenzialmente nei nuclei più piccoli. Per spiegare tale fenomeno è stata fatta l'ipotesi di una fase di accrescimento nucleare in assenza di sintesi di DNA.

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On the Mechanism of the Stimulating Action of Ribonucleic Acids on the Antibody Responses

The presence in lymphoid tissues and blood serum from immunized animals of a messenger RNA playing an important role in the antibody synthesis has been reported[1-5]. It has also been pointed out by several authors that there is an aspecific stimulating action of nucleic acids and nucleic acid-rich substances on antibody responses to different antigens[6-11]. In the following experiments we shall try to elucidate the mechanism of this aspecific adjuvant action and to show the possible differences between it and that of specific messenger RNA.

Rabbits weighing about 2 kg, fed on a standard diet and subdivided into several groups which were treated with different antigens, were used. Antigens were guinea-pig red blood cells (RBC), horse serum albumin and globulins (HSA, HSG) and bovine serum albumin (BSA) respectively. Nucleic acids used were: RNA from yeast (Schwartz) purified by precipiting and washing with 66% ethanol in the cold, and rabbit's liver RNA obtained with a phenol procedure (tissue was homogenized in ice with 7 vol. of acetate buffer pH 4.5 and then extracted and purified through several steps as described elsewhere[5]); the RNA recovery was about 500 mg/70 g fresh tissue.

RNA and antigens were injected in the following ways: (1) RNA and antigens were incubated for 30 min at 37 °C in separate tubes and then injected or separately or mixed immediately before injection. (2) RNA and antigen were mixed together, incubated for 30 min at 37 °C and then the mixture was injected. The whole cycle of immunization consisted of 4 i.v. injections made at 7 days intervals; circulating antibody titres were determined before each injection and 4 days after the last one, by the usual hemoagglutination technique for RBC and the zonal precipitation technique for soluble antigens.

RNA and soluble antigens were analysed both separately and after incubation together by paper and Tiselius electrophoresis (Perkin Elmer App. mod. 38 A)

with veronal buffer pH 8.6. RNA and proteins estimations were made by the SCHMIDT and THANNHAUSER and the FOLIN and CIOCALTEAU technique[12,13] respectively.

The data concerning the influence of RNA on the rate of immunization by several antigens are shown in Table I: the antibody response of animals immunized with soluble antigens (HSA, HSG, BSA) preincubated with RNA both from homologous liver and from yeast, was sensibly higher than that of animals immunized with antigen alone or with antigen and RNA injected separately without being preincubated together. On the contrary, with guinea-pig RBC, even after preincubation of this type of antigen with RNA, we were unable to obtain a sensible increase in antibody responses.

To ascertain whether preincubation would give rise to the formation of a 'complex' between RNA and antigen, different procedures were used, according to the nature of the antigen: in the case of particulate antigen, i.e. guinea-pig RBC, these were put in contact with a known dose of RNA for 30 min at 37 °C and the latter was estimated in the supernatant fraction before and after incubation: as can be seen in Table II, after incubation, RNA content of supernatant fraction was decreased by about 25%. In the case of a soluble antigen, i.e. BSA, polistirene latex particles were coated with the antigen by being kept 24 h at 2 °C and 24 h at room temperature, then the particles were washed twice by centrifuging at 15,000 *g* for 30 min to remove every trace of antigen remained in suspension and then incubated for 30 min at 37 °C in the presence of a known amount of yeast RNA; at this point the latex particles were centrifuged again and the RNA sedimented with the particles and what remained in suspension was estimated. As can be seen in Table II, about 37% of added RNA had been adsorbed by latex particles coated with BSA. A control made using the same latex particles not coated with the antigen showed only a minimal absorption of RNA (Table II).

An attempt to isolate the 'complex' between RNA and antigen BSA was made by paper electrophoresis of RNA-BSA preincubated together and, as a control, of RNA and BSA alone: results given in Table III show that only a small part of RNA was found in the albumin zone after migration, while the main part of it showed a rate of migration higher than albumin: one may assume that the

[1] M. FISHMAN and F. L. ADLER, J. exp. Med. *117*, 595 (1963).
[2] E. P. COHEN, Nature *213*, 462 (1967).
[3] L. MICHELAZZI, G. NANNI, I. BALDINI and A. NOVELLI, Experientia *20*, 447 (1964).
[4] L. MICHELAZZI, A. NOVELLI, G. NANNI and I. BALDINI, Experientia *20*, 703 (1964).
[5] L. MICHELAZZI, A. NOVELLI, I. BALDINI, U. M. MARINARI and G. NANNI, Sperimentale *115*, 275 (1965).
[6] B. N. JAROSLOW and W. H. TALIAFERRO, J. infect. Dis. *98*, 75 (1956).
[7] B. N. JAROSLOW and W. H. TALIAFERRO, J. infect. Dis. *107*, 341 (1960).
[8] F. PARONETTO and H. POPPER, Proc. Soc. exp. Biol. Med. *116*, 1060 (1964).
[9] J. F. MOWBRAY and D. C. HARGRAVE, Immunology *11*, 413 (1966).
[10] J. F. MOWBRAY and J. SCHOLAND, Immunology *11*, 421 (1966).
[11] B. A. ASKONAS and J. M. RHODES, Nature *205*, 470 (1965).
[12] G. SCHMIDT and S. J. THANNHAUSER, J. biol. Chem. *161*, 83 (1945).
[13] O. FOLIN and V. CIOCALTEAU, J. biol. Chem. *73*, 627 (1927).

Table I. Enhancement of antibody response to different antigens by RNA from different source in various experimental conditions

Antigen	Type RNA	Preincubation of RNA with antigen	Antibody titres at various days of immunization 7	14	21	25	No. animals
HSA	rabbit liver	without (mixed just before injection)	160	640	2560	5120	4
HSA (control)	–	–	40	160	640	1280	3
HSG	rabbit liver	without (mixed just before injection)	80	2560	5120	10240	2
HSG (control)	–	–	40	320	1280	2560	3
HSG	yeast	without (mixed just before injection)	640	1280	5120	10240	2
HSG (control)	–	–	80	320	1280	2560	3
HSG	yeast	30 min at 37 °C	640	5120	20480	40960	5
HSG (control)	–	–	80	320	640	2560	3
BSA	yeast	30 min at 37 °C	1280	5120	20480	40960	5
BSA (control)	–	–	40	160	1280	2560	3
BSA	yeast	without (injected separately)	80	320	1280	5120	5
BSA (control)	–	-	40	160	1280	2560	3
Guinea-pig RBC	yeast	30 min at 37 °C	80	320	1280	2560	4
Guinea-pig RBC (control)	–	–	80	320	640	1280	4

For each injection were used: 30 mg HSA, HSG, BSA respectively or 2 ml of 50% RBC with 30 mg RNA. Values represent the average titre rounded to the nearest multiple of the titre 10.

complex formed as a result of incubation was characterized by rather weak bonds and was therefore sensitive to the action of the strong electric field applied to it in the electrophoretic system.

In accordance with the findings of several authors, we have shown the possibility of stimulating immune responses by the use of nucleic acids of different nature. With soluble antigens, the stimulating action is already evident on the primary response and continues progressively on the secondary and tertiary response; it is not dependent on the nature of RNA used, but a great importance seems to attach to the contact between RNA and antigens: in fact introducing the 2 substances separately, we can obtain only a very small increase in the antibody response, which is just a little bit higher on injecting RNA and antigen mixed together immediately before injection and attains the highest level by introducing RNA and antigen mixed together and incubated for 30 min at 37 °C.

It therefore seems likely that the greater stimulating power acquired by RNA after being incubated together with soluble antigens may be related to a 'complex' formed in vitro: data given above show that antigens, both soluble and particulate, are able to bind RNA added in vitro. It is difficult at present to explain the nature of the bond; in any case, from the electrophoretic experiments showing the different rate of migration of the 2 elements of the 'complex', it may be thought to be a rather weak physical bond, unable to bear the action of a strong electric field.

As to the mechanism of the stimulating action in the case of soluble antigens, we can suppose that the little stimulation obtained by injecting RNA separately from the antigen may be due to the nucleic acids or their components stimulating non-specifically immunocompetent cells by providing material for synthesis of structural nucleic acids which play an important role in the antibody formation. The greater stimulation obtained by preincubation of RNA in contact with the antigen is probably due to the binding occurring between them, so that RNA can be carried rapidly and directly to the cells responsible for the analysis of antigen and the elaboration of the template on which antibodies are then produced; this will result in a prompter and greater stimulation of antibody production. It is not possible to apply this hypothesis to the RBC, because we did not obtain any significant change of their immunizing power, although they are able to bind RNA added in vitro like soluble antigens; that may be determined by (1) difference of immunocompetent clones leading the synthesis of anti RBC antibodies, or (2) the complex structure of RBC antigen which prevent the formation of an immunologically active bond between RNA and structural proteins.

In any case, even if the details of the mechanism of the observed phenomena are not quite clear, such phenomena are completely different from those occurring in case of induction of a specific antibody response by a specific RNA from immunized animals, as we reported in previous papers[3-5]. As a matter of fact, the 2 phenomena show the following differences: (1) induction of antibody synthesis by a specific immune RNA takes place in complete absence of antigen; (2) circulating antibody appears almost immediately and is already detectable 12 h after a specific RNA injection, while in present experiments and in other similar[11] the induction period lasts several days; (3) antibody response induced by specific immune RNA disappears rapidly and is no longer detectable 5 days after RNA injection, while in these experiments we found circulating antibodies several days after the last RNA-antigen injection; (4) immune response following specific immune RNA is strictly specific and depends on the type of antigen used to stimulate the production of the specific immune RNA, while the phenomena described here are not dependent on the nature of the RNA used and, within certain limits, on the nature of the antigen used; in fact 2 different types of RNA from different sources are able to stimulate, at the same rate, the antibody response to at least 4 different antigens.

Table II. Absorption of yeast RNA on different antigens

Antigen	Estimation of RNA after contact with antigen for 30 min at 37 °C and successive centrifugation			
	RNA in the sediment		RNA in the supernatant	
	mg RNA	recovery	mg RNA	recovery
Guinea-pig RBC	2.5	25%	7.2	72%
Control (RBC + RNA without incubation)	0.3	3%	9.4	94%
Latex particles coated with HSA	3.7	37%	5.7	57%
Control (latex particles not coated with HSA)	0.6	6%	9.0	90%

10 mg of BSA or 1×10^{10} RBC with 10 mg of RNA were used. RBC were centrifuged at 250 *g* for 10 min and latex particles at 15,000 *g* for 30 min.

Table III. Paper electrophoresis analysis of RNA and antigen BSA

	Recovery of RNA and BSA in the					
	prealbumin zone		albumin zone		postalbumin zone	
	RNA	BSA	RNA	BSA	RNA	BSA
RNA alone	–	–	–	–	0.940	–
BSA alone	–	–	–	0.930	–	–
RNA + BSA without preincubation	–	–	0.098	0.970	0.820	–
RNA + BSA after preincubation	–	–	0.145	0.920	0.740	–

1 mg RNA and 1 mg BSA respectively were used alone and 1 mg RNA + 1 mg BSA without preincubation and after preincubation 30 min at 37 °C. Electric potentials applied to electrophoretic strips was 120 V with 0.8 mA for each strip; migration time 16 h. Estimations were made after elution of the substances with distilled water; values are expressed in mg.

Riassunto. È stata dimostrata la possibilità di intensificare la risposta immunitaria verso albumine e globuline eterologhe, nel coniglio, con l'uso di acidi nucleici di differente origine; non è stato, invece, possibile ottenere un significativo aumento della risposta verso i globuli rossi eterologhi. Si discute sull'eventuale meccanismo che sta alla base di tale stimolazione a sui possibili motivi del differente comportamento dei globuli rossi eterologhi.

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