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STIMULATION OF THE IMMUNE RESPONSE *IN VIVO* BY DIFFERENT NUCLEIC ACIDS

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ABSTRACT

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The number of haemolytic plaque forming cells (PFCs) in the spleens of normal mice could not be increased by the injection of nucleic acids. However, when nucleic acids were injected into mice simultaneously with a priming dose of 3×10^6 sheep erythrocytes (SRBCs) an appreciable stimulatory effect was observed. The same dose of SRBCs did not result in an increase in PFCs when injected alone.

Nucleic acids at a concentration of approximately 1,5 mg per mouse resulted in an optimal stimulation of the immune response. Treatment of the nucleic acids by ribonuclease and deoxyribonuclease essentially eliminated the stimulatory effect of the RNA and DNA respectively whereas incubation with trypsin did not. Administration of pure single stranded RNAs from the livers and spleens of both normal and immunized mice resulted in a greater increase in the number of PFCs than nucleic acids from other sources. The stimulation of PFCs by RNAs from immunized mice was slightly greater than by RNAs from non-immunized mice. The immune response was also activated if the nucleic acids and the priming dose of antigen were not injected simultaneously.

INTRODUCTION

In a recent review, Gottlieb (1973) stated that the subject of RNAs and immunity is a confusing one to both the biochemist and the immunologist. The reason for this, as he correctly stated, is the conflicting evidence that has been obtained in both *in vivo* and *in vitro* studies on the specificity of the transfer of immunity by RNA. There are three hypotheses regarding the role played by nucleic acids in the immune response: (i) the transfer of "processed" antigen, attached to RNA, from phagocytes to lymphocytes resulting in the activation of the latter; (ii) the synthesis of specific antibodies mediated through specific messenger RNAs for the light and heavy immunoglobulin polypeptide chains; (iii) non-specific adjuvant-like stimulation of the immune response by nucleic acids. The latter aspect has received particular attention in this investigation.

Taliaferro & Jaroslow (1960) and Jaroslow (1968) injected SRBCs together with various nucleic acid preparations into x-irradiated rats. The immune response was partially restored by either degraded RNA or DNA, but not by the intact nucleic acids or nucleotides. Braun & Nakano (1965) confirmed that enzymatic digest of DNA increased the numbers of haemolytic PFCs after immunization with SRBCs. Enzymatic digests of RNA and oligodeoxyribonucleotides were not capable of stimulating the immune response unless specific antigen was injected concurrently. Similarly, ribonucleotide homopolymers stimulated the antibody response (Braun & Nakano, 1967; Johnson & Johnson, 1971). According to Schmidtke & Johnson (1971) equimolar complexes of synthetic polyribonucleotides of adenylic acid and uridylic acid or inosinic acid and cytidylic acid had an adjuvant effect on antibody synthesis in mice. It was postulated that the induction period of the immune response had been shortened by the nucleic acids.

In the present study the effect of different nucleic acids on the immune response of mice to low priming doses of SRBCs was investigated.

MATERIAL AND METHODS

Animals

Ten-week old male albino mice from the local colony were used.

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Nucleic acid preparations

RNA from mouse tissues. The hot phenol procedure which was employed was adapted from the method described by Scherrer & Darnell (1962). Pools of livers or spleens from 30–90 non-immunized mice or mice immunized 4 days previously with 2×10^9 freshly washed SRBCs/mouse were homogenized in cold balanced salt solution (BSS) (Mishell & Dutton, 1967). After centrifugation the packed cells were ruptured in 60–160 ml 0,01 M acetate buffer, containing 0,05% polyvinyl sulphate (PVS) and 0,5% sodium dodecyl sulphate, at pH 5. Hot, freshly distilled, water-saturated phenol was added to the ruptured cells, the suspension heated to 55 °C in a water bath at 65 °C and shaken vigorously for 3 min. After cooling to 4 °C, phase separation was achieved by centrifugation at 4 000 *g* at 2 °C for 10 min. Two more phenol extractions were carried out on the aqueous phase followed by 4 extractions with redistilled diethyl ether. RNA was then obtained from the aqueous phase by 2 successive precipitations with 2 volumes of 96% ethanol in 0,1 M NaCl at —20 °C. Single stranded RNA was obtained by an overnight precipitation in 1,0 M NaCl at 4 °C followed by an ethanol precipitation at —20 °C. The final precipitate was dissolved in 0,95% (m/v) sterile NaCl solution containing 0,05% (m/v) PVS and suitable aliquots stored in liquid nitrogen until further use. The purity of the RNA preparations was determined spectrophotometrically (Oellermann, 1974) and the A_{260}/A_{280} ration was always >2.

The nucleic acids obtained from the different mouse tissues are listed in Table 1.

TABLE 1 Sources of nucleic acids used

Description of nucleic acids	Abreviation used in text
Liver RNA from non-immunized mice	NL-RNA
Liver RNA from SRBC immunized mice	ImmL-RNA
Single stranded liver RNA from non-immunized mice	ssNL-RNA
Single stranded liver RNA from SRBC immunized mice	ssImmL-RNA
Spleen RNA from non-immunized mice	NS-RNA
Spleen RNA from SRBC immunized mice	ImmS-RNA
Single stranded spleen RNA from non-immunized mice	ssNS-RNA
Single stranded spleen RNA from SRBC immunized mice	ssImmS-RNA
Purified yeast transfer RNA	tRNA
Total yeast RNA	Y-RNA
Calf thymus DNA	T-DNA
Micrococcus lysodeiktikus DNA	M-DNA

Commercial nucleic acid preparations. Nucleic acids of yeast, calf thymus and *Micrococcus lysodeiktikus** were used for comparative purposes and are also listed in Table 1. Mass-measured samples of these nucleic acids were freshly prepared in physiological saline prior to each experiment.

Enzyme treatment of nucleic acids

Approximately 5 mg of nucleic acid was incubated at 37 °C for 30 min with either 10/μg ribonuclease (RNase), 40/μg deoxyribonuclease (DNase), 100/μg trypsin or saline. Subsequently, 10^7 SRBCs were mixed with the digest and 1,5 mg nucleic acid digest injected per mouse.

Assay of the immune response

Three mice were used per assay. SRBCs were prepared from sheep blood within 10 days of bleeding. Prior to each experiment blood was washed 3 times with BSS, the erythrocytes being separated by centrifugation at 1 000 *g* for 7 min, and diluted to the required concentration in BSS. Nucleic acids, in physiological saline, were injected intraperitoneally together with, or separately from, a priming dose of 3×10^6 SRBCs per mouse.

After 4 days mice were killed by cervical dislocation, the spleen removed aseptically into cold BSS in a petri dish and teased into small pieces with a scalpel. The pieces were further broken up by repeated, careful aspiration through a 2 mm diameter syringe needle. After allowing the larger pieces to settle, the cell suspension was carefully removed and centrifuged at 800 *g* at 4 °C for 7 min. The cell pellet was resuspended in 25 times the packed cell volume of BSS. Viable cells, which always constituted at least 98% of the total, were counted in a haemocytometer after staining with nigrosine. Serial 3-fold cell dilutions were made and direct (19S) PFCs were determined by means of the Jerne haemolytic plaque technique (Jerne, Nordin & Henry, 1963) as described by Mishell & Dutton (1967). A 0,1 ml aliquot of a prewarmed spleen cell dilution was added to 0,6 ml of a 0,5% (m/v) agarose solution in BSS, premixed with 1/10th its volume of a 7% (v/v) SRBC suspension in BSS, at 43 °C. The contents of the tube were rapidly mixed and poured onto labelled slides precoated with 0,1% (m/v) aqueous agarose. After gelling the slides were flooded with 0,4 ml of a 1 : 15 dilution of frozen guineapig serum in BSS as a source of complement. Plaques were scored after 3 h incubation at 37 °C in a humidified atmosphere.

Results

The injection of immune mouse RNA from either livers or spleens (cf. Table 1) into normal mice did not result in a significant stimulation of the immune response to SRBCs. (Oellermann, unpublished observations, 1973). It was therefore attempted to obtain a stimulation by the injection of RNA in the presence of a low priming dose of antigen or by RNA injection into antigen-primed mice. A dose of 3×10^6 SRBC per mouse did not stimulate PFCs when injected alone (Oellermann, unpublished observations, 1973), and was used throughout.

Optimum nucleic acid concentration for the stimulation of the immune response

Different concentrations of Imm L-RNA, Y-RNA or T-DNA were mixed with the constant priming dose of antigen and injected into mice. The immune response was measured 4 days later and the results are illustrated in Fig. 1.

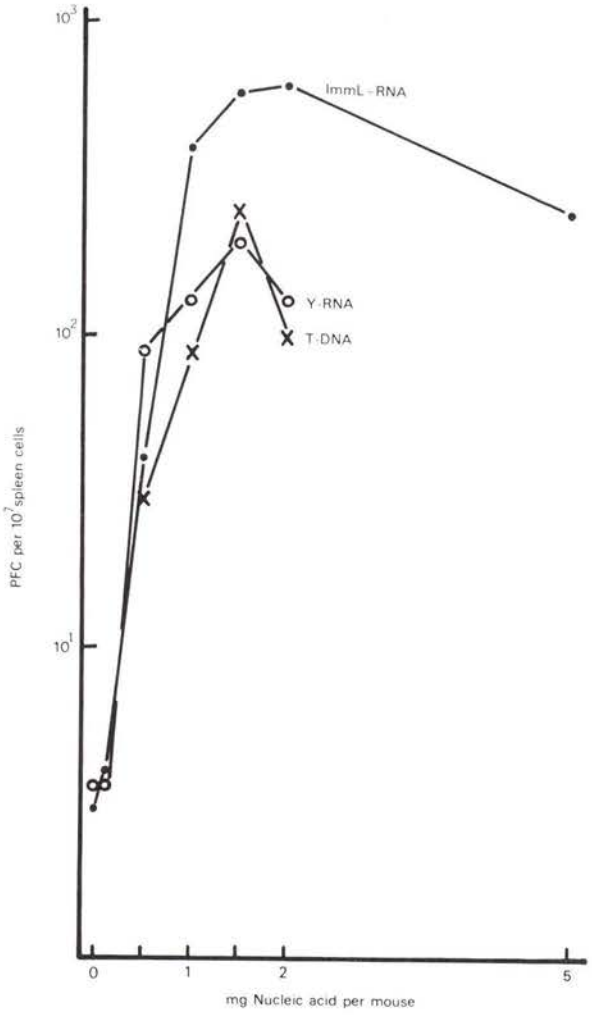

FIG. 1 Stimulation of the immune response by nucleic acids at different concentrations. Each point represents the mean of triplicate assays

Concentrations between 1 and 2 mg nucleic acid per mouse were found to be optimal. Higher concentrations of nucleic acids appeared to be less effective. Both Y-RNA and T-DNA were found to be stimulatory in a narrower concentration range than that for Imm L-RNA.

Influence of enzyme treated nucleic acids on the immune response

It was necessary to determine whether the stimulatory effect on the immune response was due to the nucleic acid or to a contaminant thereof. Only slight impurities, most probably of protein origin, could be detected spectrophotometrically in the T-DNA and Y-RNA preparations. The mouse RNA preparations employed were apparently free of protein or glycogen contaminants, but a slight contamination by DNA could not be excluded.

The influence of nucleic acids digested with RNase, DNase and trypsin on the immune response to priming doses of SRBCs was compared with that of undigested controls. The results are presented in Table 2.

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TABLE 2 The influence of enzyme treatment of nucleic acids on the stimulation of the immune response *in vivo*

Nucleic acid	3×10^6 SRBCs per mouse	PFCs/10^7 spleen cells			
		Treatment			
		Saline	RNase	DNase	Trypsin
ImmL-RNA..	+	510	27	440	579
Y-RNA.....	+	230	52	190	210
T-DNA.....	+	325	275	84	305
None.....	+	34	27	29	17
None.....	−	8	12	17	4

These data indicate that treatment of the nucleic acids by RNase and DNase practically eliminated the stimulatory effect of the RNA and DNA respectively. Incubation with trypsin did not result in a significant change of the stimulatory effect observed in the saline control groups. It must be mentioned, however, that occasionally treatment of DNA with DNase did not decrease but in fact produced a greater stimulation of the immune response than DNA alone.

Influence of different types of nucleic acids on the immune response

As both RNA and DNA were found to stimulate the immune response when mixed with a priming dose of SRBCs, a number of nucleic acids from various sources were compared. The results are presented in Fig. 2.

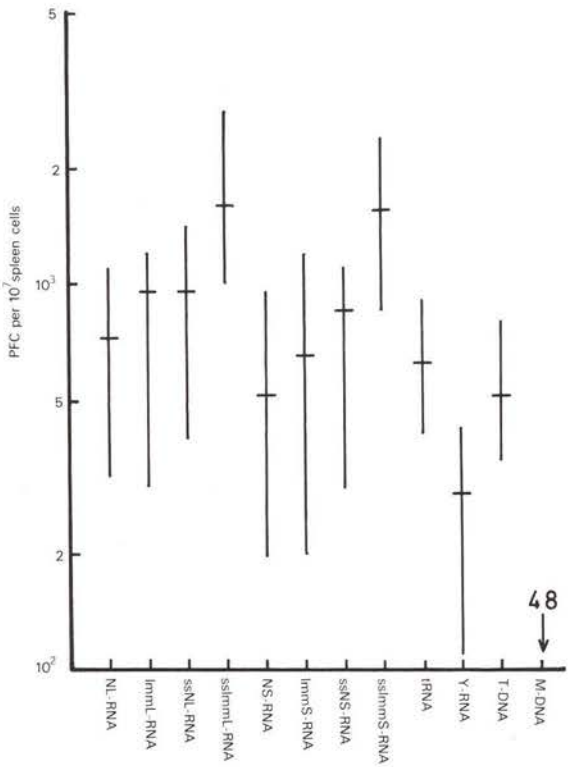

FIG. 2 The influence of different nucleic acids on the immune response in antigen primed mice. The vertical bar represents the variation between different experiments on the individual nucleic acids. The horizontal bar is the mean of 8 assays on NS-RNA, 7 each on ImmL-RNA and ImmS-RNA, 6 each on NL-RNA and ssNS-RNA, 5 each on ssNL-RNA, ssImmL-RNA and ssImmS-RNA, 4 on T-DNA, 3 on Y-RNA, 2 on tRNA and 1 on M-DNA

The range of values obtained indicates that a considerable variation was recorded between different experimental determinations of the immune response elicited by each of the individual nucleic acids. This variability made an interpretation of the results difficult. The ability of the nucleic acids to stimulate the immune response decreased in the following order: ss Imm L-RNA, ss Imm S-RNA > ss NL-RNA, ss NS-RNA, Imm L-RNA > NL-RNA, Imm S-RNA, tRNA > NS-RNA, T-DNA > Y-RNA > M-DNA $\simeq$ Controls. It is also apparent that no marked differences exist between liver and spleen RNA of comparable purity (ss NL-RNA: ss NS-RNA; ss Imm L-RNA: ss Imm S-RNA). Single stranded RNA preparations produced a slightly greater stimulation compared with the alcohol-precipitated RNA preparation from the same organ (ss NL-RNA: NL-RNA; ss Imm L-RNA: Imm L-RNA; ss NS-RNA: NS-RNA; ss Imm S-RNA: Imm S-RNA). Finally the stimulatory effect on the immune response elicited by the single-stranded immune RNA of both the liver and spleen was on the average greater than any other response observed.

Stimulation of the immune response by nucleic acids in antigen primed mice

In the foregoing experiments, nucleic acids were added to the priming dose of antigen before injection into non-immunized mice. To determine whether contact between the nucleic acid and the antigen was a prerequisite for the stimulation of the immune response, mice were primed with antigen on successive days prior to injection with nucleic acid. Four days after the nucleic acid injection the immune response was assayed. The results are presented in Table 3.

TABLE 3 Stimulation of the immune response by nucleic acids in antigen primed mice

Mice primed prior to nucleic acid injection (Days)	Nucleic acid injected on Day 0	PFCs/10^7 spleen cells[a]
4.....	None.....	7
	L/S-RNA[b].....	12
3.....	None.....	24
	L/S-RNA.....	96
2.....	None.....	29
	L/S-RNA.....	320
	Y-RNA.....	80
	T-DNA.....	54
1.....	None.....	19
	L/S-RNA.....	81
	Y-RNA.....	26
	T-DNA.....	27
0.....	None.....	12
	L/S-RNA[c].....	580
	L/S-RNA[d].....	169
	Y-RNA[d].....	30
	T-DNA[d].....	40

[a]Means of 6 experiments
[b]Liver and spleen RNA from normal and SRBC-immunized mice used in different experiments and the means determined
[c]RNA mixed with 3×10^6 SRBCs before injection
[d]Nucleic acid injected 1 h after priming

Optimal stimulation was observed in those mice primed 2 days prior to injection with nucleic acids. The stimulation in primed animals was, however, invariably less pronounced than that observed when RNA was mixed with the priming dose of antigen before injection. Both Y-RNA and T-DNA only had a minimal effect on SRBC-primed mice.

Discussion

In this investigation all nucleic acids, except M-DNA, if used at optimal concentrations, caused a 10–100 fold increase in the numbers of PFCs compared to control values observed with low priming doses of antigen alone. It is noteworthy that the pure single stranded RNA preparations from livers and spleens resulted in a greater stimulation of PFCs than the alcohol precipitated RNAs from the same organs. Whether the difference could be ascribed to the presence of contaminants or to breakdown of RNA in the preparation was, however, not determined. Of greater importance, probably, was the observation that the stimulation of the immune response by single stranded RNAs from both immunized and non-immunized mice was greater than by nucleic acids from other sources except for Imm L-RNA. Practically no difference was observed between comparable RNA preparations from either liver or spleen. These observations indicate that nucleic acid preparations from species homologous to the assay system cause a greater stimulation of the immune response than heterologous nucleic acids. This was also observed when nucleic acids were injected into mice previously primed with SRBCs. Further investigation is required to confirm the validity of this statement.

The major contribution by nucleic acids to the stimulation of the immune response appeared to be non-specific. Although there was considerable overlap, the response to RNAs from immune mice, nonetheless tended to be greater than that to comparable RNAs from non-immune mice. No inbred mice were available for this study. It is, however, probable that their use would facilitate a clearer distinction between specific and non-specific effects of these nucleic acids on the immune response.

Occasionally treatment of DNA with DNase did not decrease but in fact produced a greater stimulation of the immune response than DNA alone. The reasons for this observation are not clear, but Braun & Nakano (1965) have reported that DNase digests of calf thymus DNA stimulated the number of PFCs in mouse spleen cells whereas the deoxyribonucleotides did not.

Also of interest was the observation that nucleic acids injected separately from the priming dose of antigen resulted in a stimulation of the immune response. Although the effect was less pronounced, it is clear that direct contact between the nucleic acid and antigen before injection is not a prerequisite for the expression of this phenomenon. This can possibly be explained by dilution and breakdown of the nucleic acid before it reaches the site of action. RNA therefore appears to be effective in the stimulation of the immune response of mice previously primed with low doses of antigen. Schmidtke & Johnson (1971), however, observed an influence on the early phase of the immune response which was ascribed to an increased supply of oligonucleotides and a shortening of the induction period by RNA was postulated. This apparent difference in results requires further study and the mechanism of the immune response remains to be determined.

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