INHIBITORY EFFECT OF RIFAMYCIN DERIVATIVES ON IMMUNOGENIC RNA

Sir:

It was found in 1961 by this laboratory that cellular immunity was transferable from immune to non-immune macrophages through a transfer agent(TA) of ribonucleic acid(RNA) nature, that was extractable with phenol from the cell culture medium of immune macrophages or from immune macroghages themselves.[1~5]

Similarly, it was shown that RNA preparations, extracted from the spleens of animals immunized with various antigens[6~10] or from the peritoneal exudate cells incubated with various antigens[11~15], are capable of transferring immune response both *in vitro* and *in vivo* to non-immunized cells.

We have demonstrated that the immune RNA preparations extracted from the spleens of animals immunized with either diphtheria toxoid or *Salmonella* flagella could induce a characteristic secondary antibody formation response by injection of a small amount of antigen into animals previously injected with the corresponding immune RNA[8,9,13,16].

It was found also that immunity (primary cellular antibody formation) against either *Salmonella* infection or *Salmonella* flagella[5,17,18] or a characteristic secondary antibody formation response was transferred serially and passively through immune RNA preparations. These results strongly suggest that immune RNA is functional *per se*, replicated actively in recipient cells and does not contain antigen or fragment thereof. We deal here with the mechanisms responsible for the replication of immune RNA in recipient cells, based on experiments examining the enzymatic incorporation of H^3-UTP into RNA.

Salmonella tennessee flagella were prepared as described previously[10] and used as the antigen. Each mouse was immunized with an intravenous injection of 20 μg of the flagella preparation in 0.1 ml saline. Spleens were removed from the mice 72 hours after immunization and used as the source of immune (*i*)-RNA preparation. The RNA preparation was extracted with phenol as described previously[10]. The spleens of normal mice were used as the source of normal(*n*) RNA. The enzyme extract (*i*-EXT) was prepared from spleens of immunized mice by the method of WILLIS *et al.*[20] A similar extract (*n*-EXT) was prepared from normal spleens.

One ml of the reaction mixture contained 20 μmoles Tris-HCl (pH 7.5), 10 μmoles Mg-acetate, 4 μmoles $MnCl_2$, 2 μmoles dithiothreitol, 1 μmole each of GTP, ATP and CTP, and 10 μCi H^3-UTP (The Radiochemical Center, England), 100 μg of *i* (or *n*)-RNA, and 300 μg of *i* (or *n*)-EXT. At the appropriate incubation time at 37°C, 400 μg of bovine serum albumin and 3 ml of 10 % trichloroacetic acid(TCA) containing 0.02 M sodium pyrophosphate were added to 0.2 ml of the reaction mixture. After standing for 1 hour at 0°C, the precipitate was filtered through a glass filter (GF/C, Whatman), washed with 30 ml of cold 10 % TCA, dried and dissolved in 10 ml of omnifluortoluene solution. The sample from the glass filter was counted by a liquid scintillation counter.

Actinomycin D was kindly supplied from Merck, Sharp & Dohme Research Laboratory. Mitomycin C and bleomycin A_2 were supplied through the courtesy of Kyowa Hakko Co., Ltd., Tokyo, and Nihon Kayaku Co., Ltd., Tokyo, respectively. Two derivatives of rifamycin, *i.e.*, 2, 6-dimethyl-4-benzyl-4-demethyl rifampicin (DBD-RF) and 3-formyl rifamycin SV O-*n*-octyloxime (FR-SV) were kindly supplied by Gruppo Lepetit, Research Laboratories, Milano, Italy.

As shown in Table 1, the incorporation of H^3-uridine into the acid-insoluble fraction was demonstrated in the complete reaction mixture containing both *i*-RNA and *i*-EXT. By contrast, incorporation was not found in the reaction mixture without RNA as a template. Use of *i*-RNA gave almost 3 times more incorporation than did use of *n*-RNA. In the reaction mixture containing either (*i*-RNA and *n*-EXT) or (*n*-RNA and *i*-EXT), only a minimal increase in the incorporation of H^3-uridine was demonstrated when compared with controls containing no added RNA. The template activity of *i*-RNA

Table 1. Incorporation of H^3-uridine into RNA

RNA	EXT	H^3-UTP incorporation (cpm)
i-RNA	*i*-EXT	3,890
	n-EXT	660
n-RNA	*i*-EXT	1,410
	n-EXT	506
—	*i*-EXT	403
	n-EXT	409
i-RNA treated with RNase*	*i*-EXT	525
i-RNA treated with DNase*	*i*-EXT	3,730
i-RNA treated with elevated temperature**	*i*-EXT	620

The complete reaction mixture, RNA and EXT; see the text. Both *i*-RNA and *i*-EXT were prepared from mice immunized with *Salmonella* flagella. After 3 hours of incubation at 37℃, the radioactivity in the acid-insoluble fraction from 0.2 ml sample was counted. Ribonuclease(RNase) and deoxyribonuclease (DNase) were purchased from Worthington Biochemical Co., USA. RNase was treated at 80℃ for 30 minutes before use in order to remove any residual DNase activity.

* A hundred μg of *i*-RNA was pretreated with either RNase (50 μg) or DNase (50 μg) at 37℃ for 10 minutes and was added to each reaction mixture without removal of the nucleases.

** *i*-RNA was treated with 0.5 N NaOH in boiling water for 10 minutes and its pH was adjusted to 7.5 using 0.5 N HCl after cooling. The treated *i*-RNA equivalent to 100 μg of the original *i*-RNA was added to the reaction mixture.

was greatly decreased when it was either treated with RNase or subjected to an elevated temperature at an alkaline pH. In contrast treatment with DNase did not affect template activity of *i*-RNA.

The inhibitory effect of antibiotics on the incorporation of H^3-uridine into RNA was examined by using mitomycin C, actinomycin D, bleomycin A_2, RF-SV and DBD-RF. As shown in Table 2, the first three drugs did not have any inhibitory effects on the incorporation of H^3-uridine into RNA. Therefore, it was concluded that the RNA synthesis observed was due to the RNA itself as a template and not to any traces of DNA. The two derivatives of rifamycin, that have been shown to inhibit an RNA-dependent DNA polymerase activity[21,22], possessed an inhibitory effect on the incorporation of H^3-uridine into RNA.

Our studies described herein indicated that an RNA replicase activity was to be found

Table 2. Effect of antibiotics on the incorporation of H^3-uridine into RNA

Drug	Amount added (μg/ml)	Incorporation of H^3-UTP*	
		cpm	In percent of control
—	—	4,024	100
Mitomycin C	1	3,823	95
Actinomycin D	1	3,702	92
Bleomycin A_2	100	4,437	110
	50	4,010	100
FR-SV	500	454	11
	250	776	19
DBD-RF	500	730	18
	250	1,044	26
	100	1,483	37

The complete reaction mixture, RNA and EXT; see the footnote of Table 1. Both *i*-RNA and *i*-EXT were prepared from mice immunized with *Salmonella* flagella. One hundred μg of *i*-RNA was treated with each of antibiotics at 37℃ for 10 minutes and was added to the reaction mixture without removal of the antibiotics. After 3 hours of incubation at 37℃, the radioactivity in the acid-insoluble fraction from 0.2 ml sample was counted.

* A background incorporation of radioactivity, from unincubated controls, was subtracted to obtain the values listed above.

in the organs of immunized animals and this enzyme could induce RNA synthesis using *i*-RNA as a template. It was shown further that the template activity was lost by treatmet of *i*-RNA with RNase but not with DNase, and that the enzyme activity was not inhibited by addition of the nucleic acid polymerase inhibitors such as actinomycin D, mitomycin C and bleomycin A_2. These results indicated that the RNA synthesis was due to *i*-RNA itself as a template and not to possible traces of DNA.

Two derivatives of rifamycin, which had been shown to inhibit reverse transcriptase of oncogenic RNA viruses[21,22], have also shown inhibitory effects on the *i*-RNA dependent DNA polymerase and the *i*-RNA dependent RNA replicase in the immune response. These results suggest a new research problem to determine if there are any immunosuppressors.

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