INHIBITORY EFFECT OF ANTIBIOTICS ON THE REVERSE TRANSCRIPTASE IN IMMUNE RESPONSE

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, which was extracted from cell culture medium of immune macrophages[1] or extracted with phenol from the peritoneal exudate cells, spleen and lymph nodes of immunized animals[2~5]. It was demonstrated also that the immune (*i*) RNA preparation could induce a characteristic secondary antibody formation response by injection of a small amount of antigen into animals previously injected with the corresponding *i*-RNA[6~9].

It was found that immunity (primary cellular antibody formation) against either *Salmonella* infection or *Salmonella* flagella[2,10,11] or a characteristic secondary antibody formation response[12] was transferred serially and passively through *i*-RNA preparations.

Based on these findings, we could demonstrate an *i*-RNA replicase activity in the spleens of animals immunized with various antigens[13,14]. Our studies indicated also that a reverse transcriptase activity was found in the organs of immunized animals and this enzyme could induce DNA synthesis using *i*-RNA as a template[13]. This paper deals with the inhibitory effect of antibiotics on the reverse transcriptase in the immune response.

Salmonella tennessee flagella and f_2 phage were used as antigens. White rabbits weighing about 2.5 kg were purchased from a farm. For immunization, 50 μg of flagella in saline was injected into each of both hind foot pads and 100 μg of flagella was injected into its ear vein. A rabbit received injections of 10^{11} of f_2 phage in saline into each of its two hind foot pads and 2×10^{11} of f_2 phage into its ear vein. Rabbits were sacrificed by bleeding 3 days after injection. Spleens were used as the source of *i*-RNA preparations. Spleens of normal rabbits were used as the source of normal RNA (*n*-RNA).

Popliteal lymph nodes were collected from the hind feet of an immunized rabbit. The lymph nodes were teased and cell suspensions were obtained by straining through a stainless steel mesh and washed with saline by centrifugation. The enzyme extract was prepared from the lymph node cells by the method of GALLO *et al.*[15] The supernatant, after centrifugation at $105,000\times g$ for 60 minutes, was used as a crude enzyme extract (*i*-EXT) and the protein concentration was adjusted to 1 mg/ml.

RNA-dependent DNA polymerase (reverse transcriptase) activity was assayed by the method of GALLO *et al.*[15] One ml of the reaction mixture contained 50 μg of *i*-RNA, 300 μg of enzyme protein, 50 μmoles of Tris-HCl (pH 8.3), 6 μmoles of magnesium acetate, 20 μmoles of dithiothreitol, 60 μmoles of NaCl, 0.8 μmole each of dATP, dCTP and dGTP, and 3 μCi C^{14}-thymidine triphosphate (The Radiochemical Center, England). The mixture was incubated at 37°C. Following incubation, 2 volumes of cold 0.08 M Na-pyrophosphate containing 400 μg of calf thymus DNA/ml was added to the mixture and then trichloroacetic acid (TCA) was added to a final concentration of 5%. The precipitate was washed with cold 5% TCA on a glass fiber filter (GF/C, Whatman), dried and dissolved in 10 ml of toluene containing 0.4% PPO and 0.01% POPOP. The sample was counted using a liquid scintillation counter.

Deoxyribonuclease(DNase) and ribonuclease (RNase) were purchased from Worthington Biochem. Co., USA. Actinomycin D and mitomycin C were kindly supplied from Merck, Sharp & Dohme Research Lab., Rahway, USA and Kyowa Hakko 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 C^{14}-thymidine into the acid-insoluble fraction was demonstrated in the reaction mixture containing both *i*-RNA and *i*-EXT. In contrast, the incorporation was greatly

Table 1. Incorporation of ^{14}C-thymidine into DNA

RNA	EXT	^{14}C-TdR incorporation (cpm)
i-RNA	*i*-EXT	4,010
	n-EXT	1,100
n-RNA	*i*-EXT	1,020
	n-EXT	120
i-RNA treated with RNase*	*i*-EXT	460
i-RNA treated with DNase**	*i*-EXT	3,870
—	*i*-EXT	40
	n-EXT	78

The complete reaction mixture, RNA and EXT; see the text. Both *i*-RNA and *i*-EXT were prepared from rabbits immunized with *Salmonella* flagella. After 6-hour incubation at 37℃, the radioactivity in the acid-insoluble fraction from 0.5 ml sample was counted.

* RNase was treated at 80℃ for 30 minutes before use in order to remove any residual DNase activity. Fifty μg of *i*-RNA was pretreated with RNase (25 μg) at 37℃ for 10 minutes and was added to the reaction mixture without removal of RNase.

** One mg of *i*-RNA was pretreated with DNase (100 μg) at 37℃. After 30-minute incubation, DNase was removed by extraction with phenol and 50 μg of DNase-treated *i*-RNA was added to the reaction mixture.

decreased in the reaction mixture without RNA preparations even with *i*-EXT. A slight increase in the C^{14}-thymidine incorporation was seen in the reaction mixture containing (*i*-RNA and *n*-EXT) or (*n*-RNA and *i*-EXT). The reaction was greatly retarded by treatment of *i*-RNA with RNase but not after treatment with DNase.

The inhibitory effect of antibiotics on the incorporation of C^{14}-thymidine into DNA was examined by using mitomycin C, actinomycin D, rifampicin, FR-SV and DBD-RF. As shown in Table 2, both FR-SV and DBD-RF showed inhibitory effects on the incorporation of C^{14}-thymidine into DNA and a large amount of rifampicin (500 μg/ml) showed a slight inhibition, while mitomycin C and actinomycin D did not have any inhibitory effects on the incorporation of C^{14}-thymidine into DNA, indicating that the DNA did not act as a template while the *i*-RNA did.

The reverse transcriptase could induce DNA synthesis only by using *i*-RNA as a template and the enzyme activity was demonstrated (or enhanced) by stimulation with the corresponding antigen. Similarly, the RNA-dependent RNA replicase was demonstrated (or enhanced) by stimulation with

Table 2. Effect of antibiotics on the incorporation of ^{14}C-thymidine into DNA

Drug	Amount added (μg/ml)	C^{14}-TdR incorporation*	
		cpm	in perceet of control
—	—	4,010	100
Mitomycin C	1	4,245	105
Actinomycin D	1	3,856	96
Rifampicin	500	615	15
	200	2,205	54
DBD-RF	200	532	13
	100	440	10
FR-SV	200	21	0.5
	100	305	7.6

Both *i*-RNA and *i*-EXT were prepared from rabbits immunized with *Salmonella* flagella. Each of antibiotics was added to the reaction mixture and incubated at 37℃. Details, see the footnote of Table 1.

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

the corresponding antigen which had been used for the production of *i*-RNA. The problem of whether or not a stimulation with antigen is needed for the production of these enzymes (or an enhancement of their activity) in the immune response, will be investigated in the near future using germ-free animals.

Two derivatives of rifamycin, which had been shown to inhibit reverse transcriptase of oncogenic RNA viruses (unpublished observation), have also inhibitory effects on the *i*-RNA dependent DNA polymerase and the *i*-RNA dependent RNA replicase[14] in the immune response. These results suggest a new research problem to determine if there are any immuno-suppressors.

Template specificity of reverse transcriptase and properties of produced DNA should be examined after the purification of the enzyme.

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SATONORI KURASHIGE
SUSUMU MITSUHASHI

Department of Microbiology,
School of Medicine,
Gunma University, Maebashi,
Japan

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References

1) MITSUHASHI, S. & K. SAITO : *In vitro* transfer of cellular immunity of mouse phagocytes in experimental salmonellosis. J. Bact. 84 : 592～593, 1962
2) SAITO, K.; I. SATO, T. TANAKA & S. MITSUHASHI : Some properties of the transfer agent(TA) which confers cellular immunity to the mouse mononuclear phagocytes. Proc. Japan Acad. 39 : 691～696, 1963
3) MITSUHASHI, S.; K. SAITO, N. OSAWA & S. KURASHIGE : Experimental salmonellosis. XI. Induction of cellular immunity and formation of antibody by transfer agent of mouse mononuclear phagocytes. J. Bact. 94 : 907～913, 1967
4) MITSUHASHI, S.; S. KURASHIGE, M. KAWAKAMI & T. NOJIMA : Transfer agent of immunity. I. Immune ribonucleic acid which induces antibody formation to *Salmonella* flagella. Jap. J. Microbiol. 12 : 261～268, 1968
5) OSAWA, N.; S. KURASHIGE, M. KAWAKAMI & S. MITSUHASHI : Experimental salmonellosis. XII. *In vitro* induction of cellular immunity by immune ribonucleic acid. Jap. J. Microbiol. 12 : 479～488, 1968
6) KURASHIGE, S.; K. KITAMURA, K. AKAMA & S. MITSUHASHI : Transfer agent of immunity. IV. Antibody formation against diphtheria toxin by an immune ribonucleic acid fraction. Jap. J. Microbiol. 14 : 41～47, 1970
7) YAMAGUCHI, N.; S. KURASHIGE & S. MITSUHASHI : Antibody formation against *Salmonella* flagella by an immune ribonucleic acid fraction. J. Immunol. 107 : 99～103, 1971
8) FURUHATA, T. & S. IKEMOTO : Immunological studies on the human spleen RNA as a transfer agent in immunity. Kriminalistik u. Forenische Wissenschaften 6 : 57～62, 1971
9) KITAMURA, K.; S. KURASHIGE & S. MITSUHASHI : Dose effect of immune ribonucleic acid fraction on antibody formation. Jap. J. Microbiol. submitted for publication, 1972
10) SAITO, K.; S. KURASHIGE & S. MITSUHASHI : Serial transfers of immunity through immune RNA. Jap. J. Microbiol. 13 : 122～124, 1969
11) SAITO, K.; N. OSAWA & S. MITSUHASHI : Transfer agent of immunity. VI. Serial passive transfers of cellular immunity to *Salmonella* infection by immune ribonucleic acid. Jap. J. Microbiol. 15 : 159～168, 1971
12) KURASHIGE, S. & S. MITSUHASHI : Serial passive transfers of immune response by an immune ribonucleic acid preparation. J. Immunol. 108 : 1034～1038, 1972
13) MITSUHASHI, S.; K. SAITO & S. KURASHIGE : The role of RNA as amplifier in the immune response. Presented at the symposium on "RNA and the immune response". The New York Academy of Sciences, April 19～21, 1972. Ann. New York Acad. Sci. in press 1972
14) SAITO, K. & S. MITSUHASHI : Inhibitory effect of rifamycin derivatives on immunogenic RNA. J. Antibiotics 25 : 477～479, 1972
15) GALLO, R. C.; S. S. YANG & R. C. TING : RNA dependent DNA polymerase of human acute leukemic cells. Nature 228 : 927～929, 1970