Induction in vitro of Antibodies to Phage T2: Antigens in the RNA Extract Employed

Abstract. *Cells from rat peritoneal exudate incubated in vitro with phage T2 yield RNA which induces cultures of rat lymph nodes to form antibodies. Complement fixation reveals T2 head, tail, and internal protein antigens in the RNA. Inoculating the RNA into mice that had previously been stimulated primarily with whole phage T2 results in a secondary response of neutralizing antibody.*

Fishman (*1*) and Fishman and Adler (*2, 3*) have described the induction of antibody synthesis in vitro. They incubated bacteriophage T2 and rat cells from peritoneal exudate, termed macrophages, for 30 minutes at 37°C. A cell-free homogenate of these macrophages was added to rat lymph-node cells in culture. Within a week specific neutralizing antibodies to phage T2 appeared in the medium. Since ribonuclease and streptomycin rendered this homogenate inactive, it was assumed that RNA was the active factor in the homogenate. Subsequently, a diffusion chamber was inserted intraperitoneally into x-irradiated rats (*2*). This diffusion chamber was charged with rat lymph-node cells and an RNA fraction derived from rat macrophages that had been incubated with phage T2. Neutralizing antibody appeared in the serum of the rats within 6 days, but antibody formation did not occur if the RNA from macrophages was treated with ribonuclease before being inserted in the chamber. The active RNA was of relatively low molecular weight as judged by gradient centrifugation (*2, 3*). Such low-molecular-weight RNA was readily incorporated into lymph-node cells in culture (*4*). The efficacy of the preparation of RNA from macrophages in inducing antibody synthesis was ascribed either to its content of T2 antigens or of informational RNA, which of itself contained information for the synthesis of neutralizing antibody against phage T2 (*2, 3*). The studies by Fishman and Adler (*2, 3*) did not reveal T2 antigens in the macrophage-RNA preparation.

Our study has confirmed the aforementioned results. In addition, several phage antigens have been detected in the RNA that was isolated from macrophages which had been incubated with phage T2.

Table 1. Appearance of specific phage T2 neutralizing activity in cultures of rat lymph-node cells incubated with RNA from rat macrophages which had been treated with phage T2.

Macrophage source of RNA	Inactivation (%)	
	Phage T2	Phage T5
Untreated	10	10
T2-treated	52	8
T7-treated	12	10
Untreated	0	7
T2-treated	32	9

The procedures described by Fishman and Adler (*1–3*) were followed. Peritoneal macrophages (1×10^9) from nonimmunized Wistar rats were incubated with viable particles of phage T2 or T7 (5×10^6) for 30 minutes at 37°C. The macrophages were washed three times in saline and homogenized; their RNA was extracted with three portions of water-saturated phenol. The RNA was then precipitated with seven volumes of cold absolute ethanol and dissolved in tris buffer, *p*H 7.6. RNA was similarly extracted from macrophages which had been incubated with saline instead of with phage. Control and experimental preparations of RNA were incorporated into separate portions of Trowell's medium (*5*) in which normal node cells or node fragments were cultured for 8 days in an atmosphere of 75 percent nitrogen, 20 percent oxygen, and 5 percent carbon dioxide. After this, normal rat serum (1 percent by volume) was added to the culture medium, and the γ-globulins were precipitated by the addition of an equal volume of saturated $(NH_4)_2SO_4$. Neutralizing activity was assayed by the agar-overlay method (*6*), in which 0.5 ml of the γ-globulin solution was incubated with 10^3 viable particles of phage T2. Antibody to the phage was also assayed by the complement-fixation microtechnique of Wasserman and Levine (*7*).

In confirmation of Fishman's data, neutralizing activity specific for phage T2 was found when RNA from T2-treated, but not T5-treated, macrophages was used in the lymph-node cultures (Table 1). Neutralizing activity was not detected in the medium if the lymph-node cells were incubated with RNA from untreated macrophages. Similarly, antibody was not detected if phage T2 was added directly to lymph-node cells. In extension of the original observations, specific antibodies to T2 were also detected in the medium by complement fixation with particles of phage T2 but not with phage T7 particles. The optical density of the liberated hemoglobin was 0.400 or greater in all controls as well as in the tubes containing γ-globulin derived from lymph-node cultures containing RNA from untreated macrophages. In tubes containing globulins from lymph-node cultures containing RNA from treated macrophages the optical density was 0.196.

Further analysis of the interaction between macrophage and bacteriophage revealed that the RNA obtained from the macrophages which were incubated with phage T2 at 0° and at 56°C did not induce synthesis of antibodies. Treatment of the macrophage RNA with ribonuclease reduced, but did not completely abolish, its ability to induce antibody synthesis in the lymph-node system. When the extracted RNA (0.6 to 0.9 mg) was incubated with

Table 2. Detection of T2 antigens in macrophage RNA by complement fixation; 0.005 mg of RNA was employed in the assay. Different preparations were employed in each experiment. Results are given in optical density units measured at 413 mμ.

Macrophage source of RNA	Antiserum to	Optical density	
		Treated	Untreated
T2-treated	T2 (1 : 200)	0.105	0.394
T5-treated	T2 (1 : 200)	.400	.394
T2-treated	T2	.270	.382
T5-treated	T2	.375	.390
T2-treated	Internal protein of T2	.260	.400
T5-treated	Internal protein of T2	.390	.400
T2-treated	Head protein of T2 (1 : 50)	.265	.460
T5-treated	Head protein of T2 (1 : 50)	.440	.470
T2-treated (30-min incubation)	Whole T2	.332	.435
T2-treated (90-min incubation)	Whole T2	.445	.435

enzyme (100 μg) prior to addition to the cultures, inactivation of the phage by the cultures was reduced by 45 percent. The percentage of inactivation fell from 40 to 16 percent.

The results of experiments on the macrophage-bacteriophage reaction, especially the finding that increasing the period of incubation of these reagents decreased the content of T2 antigen in the RNA (Table 2, last experiment), suggested that intracellular degradation of bacteriophage was occurring within the macrophages. Therefore, a search for antigenic fragments in the RNA was initiated. A micromethod for complement fixation (*7*) revealed the presence of phage antigen or antigens in the RNA from macrophages (Table 2). Complement fixation, as determined by microtechnique, with specific antiserums to head antigens of T2 (*8*) also revealed the presence of these antigens in the RNA (Table 3). When the same RNA preparations were tested with specific antiserums to T2 internal protein (*9*) this antigen was also found. Appropriate controls with RNA extracted from macrophages which had been incubated with T5 did not yield positive complement-fixation reactions with any of these specific antiserums to T2 antigens (Table 2).

Evidence for the presence of T2 tail antigens in the macrophage RNA was provided by inoculation of the RNA into mice that had been primed previously with this phage. Several mice that were injected with RNA from T2-treated macrophages showed an anamnestic response in producing neutralizing antibody, whereas none of those injected with control RNA preparations did so (Table 3).

In several of our preparations of RNA from macrophages the ratio of ultraviolet absorption at 260 to that at 280 mμ was somewhat lower than that reported by Fishman and Adler (*2*) for their preparations. Therefore, we obtained some macrophage RNA from these investigators and were able to demonstrate T2 antigen or antigens in their preparation by complement fixation with rabbit antiserums to T2.

Thus, several antigens of phage T2 are present in macrophage RNA that was extracted after the reaction of macrophages and phage T2. The tail antigen is present in sufficient concentration and in proper configuration to be immunogenic in primed mice. The presence of internal protein, which is

Table 3. Immunogenicity of T2 macrophage RNA in mice primed with phage T2. C57BL/J mice weighing about 22 g had been injected subcutaneously with 1×10^{10} T2 phage particles 6 weeks previously. They were then challenged with 0.125 mg of RNA from phage T2 or T5, injected subcutaneously. Inactivation before and after injection is given. Serums were diluted 1 : 40 for inactivation assay. Each RNA preparation is derived from a separate experiment.

Macrophage source of challenge RNA	Inactivation (%)	
	Before booster	7 days after challenge
T2-treated	0	80
T2-treated	30	66
T2-treated	10	81
T5-treated	22	25
T5-treated	48	20

associated with the DNA within the head of the phage (*9*), as well as the finding that less antigen is present after 90 minutes of incubation than after 30 minutes, suggests that degradation of the phage particles occurs. Electron microscope studies also suggest that breakdown of phage T2 particles occurs within macrophages (*10*).

Other studies have provided further data which may be utilized to construct a tentative picture of the early events of the immune response. Franzl (*11*) found that a lysosomal fraction of spleen from mice injected with sheep red blood cells elicited a secondary response upon injection into mice that had been primed with this antigen. He suggested that red-cell antigens were present in this subcellular fraction. Askonas and Rhodes (*12*) found that macrophages degrade hemocyanin into RNA-bound antigenic fragments which are immunogenic. In fact, their data suggest that the macrophage RNA may act as an adjuvant and promote the immunogenicity of the bound antigen. In a series of studies Garvey and Campbell (*13*) presented impressive evidence for the presence of bovine serum albumin antigen or antigens complexed with liver RNA and suggested that complexed antigen was involved in the immune response. Fishman *et al.* (*4*) have found that RNA fractions with low molecular weights are taken up by lymph-node cells, presumably lymphocytes.

From our data, as well as data of the type just summarized, the following sequence of events may be suggested to occur in the macrophages. Macrophages phagocytose phage T2 particles and rapidly degrade them. The phage antigens are then bound to certain low-molecular-weight RNA's of the cell. Presumably the antigens are thereby held in native, immunogenic configuration. Within the lymph-node cells the RNA may, in addition, function as an adjuvant for the bound T2 antigens. The loss of biological activity of the macrophage RNA after treatment with ribonuclease may be attributed to the loss of one or more of these functions after its degradation.

Whether in fact the RNA-bound T2 antigen is responsible for the induction of antibody synthesis is not known. However, the presence of antigen in the RNA seems a likely source of information for this induction. The inadequacy of the RNA extraction procedure for the preparation of heavy "messenger" RNA (*14*) also argues against the presence of messenger RNA in the macrophage-RNA preparations and, therefore, against the role of such an RNA per se in the induction of antibody synthesis in this system.

H. P. FRIEDMAN*
A. B. STAVITSKY
J. M. SOLOMON†
Western Reserve University School of Medicine, Department of Microbiology, Cleveland, Ohio 44106

References and Notes

1. M. Fishman, *J. Exp. Med.* **114**, 837 (1961).
2. ——— and F. L. Adler, *ibid.* **117**, 595 (1963).
3. ———, in *Immunopathology*, 3rd International Symposium, La Jolla, California, 1963, P. Grabar and P. Miescher, Eds. (Grune and Stratton, New York, 1964), p. 79.
4. M. Fishman, R. A. Hammerstrom, V. P. Bond, *Nature* **198**, 549 (1963).
5. O. A. Trowell, *Exp. Cell Res.* **9**, 258 (1955).
6. M. H. Adams, *Bacteriophages* (Interscience, New York, 1959), pp. 450–451.
7. E. Wasserman and L. Levine, *J. Immunol.* **87**, 290 (1961).
8. F. Lanni and Y. T. Lanni, *Cold Spring Harbor Symp. Quant. Biol.* **28**, 159 (1953).
9. L. Levine, in *Immunochemical Approaches to Problems in Microbiology* (Rutgers Univ. Press, New Brunswick, N.J., 1960), p. 171.
10. R. Aronow, D. Danon, A. Shahar, M. Aronsan, *J. Exp. Med.* **114**, 837 (1961).
11. R. E. Franzl, *Nature* **195**, 457 (1962).
12. B. A. Askonas and J. M. Rhodes, *Nature* **205**, 470 (1965).
13. J. S. Garvey and D. H. Campbell, *J. Exp. Med.* **105**, 361 (1957).
14. A. Sibantani, S. R. deKloet, V. G. Allfrey, A. E. Mirsky, *Proc. Nat. Acad. Sci. U.S.* **48**, 471 (1962).
15. Supported by PHS grant AI-01865. We thank Weldon W. Harold for technical assistance and Marvin Fishman and Frank L. Adler for aid in learning techniques in their laboratories and for numerous courtesies throughout this investigation. We appreciate the gift of specific anti-internal protein and antihead antigen serums by Lawrence Levine and Frank Lanni, respectively.
* Postdoctoral fellow of the U.S. Public Health Service. Present address: Department of Microbiology, Meharry Medical College, Nashville, Tennessee.
† Lederle medical student research fellow.
18 May 1965