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Experimental Salmonellosis

XI. Induction of Cellular Immunity and Formation of Antibody by Transfer Agent of Mouse Mononuclear Phagocytes

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When mice were injected intraperitoneally with a ribonucleic acid (RNA) preparation extracted from the peritoneal mononuclear phagocytes (termed monocytes) of immunized mice, these macrophages developed cellular immunity and cellular antibodies. The peritoneal monocytes were obtained from normal mice and maintained in tissue culture bottles in a homogeneous cell population. When they were treated in vitro with an immune RNA preparation, they acquired cellular immunity, and cellular antibodies were detectable in such monocytes. These results suggest that the mononuclear phagocytic cell line constitutes a cell line responsible for antibody formation.

In preceding papers, it was reported that mice hyperimmunized with live vaccine of *Salmonella enteritidis* acquired resistance against fatal infection with a virulent strain, 116-54, of the same organism, and the mononuclear phagocytes (referred to as monocytes) of immunized mice inhibited intracellular multiplication of virulent strain 116-54 in the absence of antibody in cell culture medium, henceforth referred to as cellular immunity. In contrast, the mice immunized with killed vaccines of *S. enteritidis* 116-54, e.g., heat-killed vaccine, Formalinized vaccine, and alum-chrome vaccine (1), did not acquire such resistance against fatal infection with the same organism (16, 23, 27, 31, 34, 35, 37). It was also found that cellular immunity was transferable, from immune to nonimmune monocytes, through the transfer agent (TA) of apparently ribonucleic acid (RNA) nature, which was obtained from immune monocytes (24, 29, 30, 36, 38; S. Mitsuhashi et al., Abstr. Meeting Japan. Bacteriol. Assoc., p. 19, 1961). In previous papers (17, 28), cellular antibody detectable in abdominal monocytes, spleen, and lymph node of immunized mice was described.

In the present article, the detection of cellular antibody in the abdominal monocytes of mice treated with an immune RNA preparation is presented, and the role of monocytes in antibody formation is demonstrated.

MATERIALS AND METHODS

Experimental animals. Mice of ddN strain 5 to 7 weeks old of both sexes (raised by the Central Animal Laboratory, Gunma University), weighing 20 to 25 g, were used.

Organisms. Organisms used were virulent strain 116-54 and attenuated strain SER of *S. enteritidis.* The bacteria were cultured on agar plates or in nutrient broth for 18 hr at 37 C.

Immunization. Immunization of mice with live vaccine was reported previously (31). Briefly, mice were injected intravenously with 10^{-5} mg (dry weight) of live vaccine of attenuated strain SER. For hyperimmunization with live vaccine, mice were inoculated intravenously with 10^{-5} mg of attenuated strain SER and, 21 days later, with 10^{-7} mg of virulent strain 116-54 of *S. enteritidis.* Immunized mice were used 21 days after the last injection.

Preparation of RNA from peritoneal cells of immune mice. Starting material for the preparation was collected from the abdominal cavity of 100 immune mice 5 days after intraperitoneal injection of 1 ml of glycogen solution (0.01 mg/ml) per mouse. The collected cells were washed twice by centrifugation at 335 × *g* for 5 min in tris(hydroxymethyl)aminomethane (Tris) buffer at *p*H 8.6 which consisted of 0.25 M sucrose, 0.025 M KCl, 0.005 M $MgCl_2$, and 0.005 M Tris chloride. After homogenization for 5 min with a Teflon homogenizer, the homogenized cells were shaken for 15 min with an equal volume of water-saturated phenol in an ice bath. The aqueous phase was re-extracted twice for 5 min with an equal volume of water-saturated phenol. Phenol was removed by five extractions with washed ether. Ether was removed by bubbling nitrogen through the solution. All procedures were performed in an ice bath except for removing ether at 20 C. Normal RNA preparation was similarly extracted from peritoneal monocytes of normal mice instead of immune mice.

Treatment of mice with RNA preparations. A recipient mouse received intraperitoneal injection of the phenol-extracted RNA preparations, equivalent to 2×10^7 cells.

In vitro treatment of monocytes with RNA preparations. The monocytes, obtained from the abdominal cavity of normal mice, were washed twice with chilled Hanks basal salt solution and were resuspended in a small volume of tissue culture medium. The cell number was adjusted to 1,000 cells/ml. A 2-ml amount of cell suspension was placed in a culture bottle provided with cover glasses and incubated for 18 hr at 37 C. Monocytes adhered to the bottom. By replacing culture medium, cell contaminants, i.e., lymphocytes and polymorphonuclear phagocytes, were removed, and 98 to 100% pure cultures of monocytes were obtained. The monocytes (about 2×10^6 cells per bottle) were treated for 3 days with the RNA preparations (equivalent to 2×10^7 monocytes) which were dissolved in culture medium and incubated at 37 C. The culture medium was changed daily with fresh medium containing the RNA preparations.

Assay of cellular antibody. Peritoneal monocytes were collected from the mice which had been injected intraperitoneally with the RNA preparations 5 days before the collection. Immune transfer and immune adherence hemagglutination (IAHA; reference 32) was used for titration of cellular antibody according to a previous description (17). Similarly, the cellular antibody of monocytes was determined by the IAHA method after in vitro treatment for 3 days with the RNA preparations.

Cellular immunity. The monocytes were collected from the abdominal cavity of mice treated with the RNA preparations 5 days before the collection. Cellular immunity was examined by infecting monocytes in vitro with the virulent strain 116-54. Monocytes, obtained from the abdominal cavity, were washed twice with chilled Hanks basal salt solution and were resuspended in a small volume of tissue culture medium which consisted of 30% horse serum and 70% Hanks solution. The cells were counted with a hemocytometer, and the cell number was adjusted to 1,000 cells/ml. A 2-ml amount of cell suspension was placed in a culture chamber provided with cover glasses and incubated at 37 C. Monocytes adhered to the bottom of the culture chamber. By replacing culture medium, cell contaminants, i.e., lymphocytes and polymorphonuclear phagocytes, were removed, and 98 to 100% pure cultures of monocytes were obtained. After 13 hr of incubation, the supernatant fluid was removed and was replaced with fresh Hanks solution containing virulent strain 116-54 and 5% normal mouse serum. The multiplicity of infection of bacteria to monocytes was 6:1. After 60 min of incubation at 37 C, the supernatant fluid containing bacteria was discarded and replaced with a fresh culture medium containing penicillin (10 units/ml) and streptomycin (10 μg/ml) to inhibit the extracellular growth of bacteria. This concentration of antibiotics allowed the full growth of bacteria in normal monocytes. The culture medium was changed daily. The cover glasses to which monocytes adhered were removed from the culture bottle at appropriate time intervals after infection, were dried in air, were fixed in methanol, and were stained in Giemsa solution. The numbers of monocytes and of infected monocytes were determined microscopically. The phagocytic index was expressed by the number of infected monocytes per total number of monocytes. The number of bacteria in infected monocytes was counted microscopically by observing at least 300 infected cells in each count and was expressed as a mean of three counts. The total number of monocytes which adhered to a cover glass was counted microscopically in five microscopic fields at a magnification of 200 and was expressed as a mean of three counts. The standard deviation of cell number on each cover glass in a tissue culture bottle was calculated as 10%, and the deviation of phagocytic index was controlled so as to remain below 5%.

Cultures of normal monocytes were prepared in a similar manner. After treatment in vitro with the phenol-extracted RNA preparations for 3 days, the supernatant fluid was removed, and cellular immunity was examined by infecting monocytes with bacteria as described above.

Inhibition of bacterial growth. Antibody of immunized monocytes was transferred to live organisms of virulent strain 116-54 as described in a previous paper (17).

Briefly, equal volumes of monocyte suspension (Ab, 10^8 cells/ml) and bacterial suspension (Ag, 10^8 organisms/ml) were mixed at 30 C for 60 min in Veronal buffer containing gelatin. The incubated mixture was centrifuged at $2,600 \times g$ for 5 min to remove monocytes, and the supernatant fluid containing sensitized bacteria (Ag-Ab) was further centrifuged for 10 min at $10,000 \times g$. The sensitized bacteria (Ag-Ab) thus obtained were washed twice with Veronal buffer containing gelatin and sucrose and were suspended in the same buffer to make a suspension of about 10^8 organisms/ml. A 0.8-ml amount of the suspension of sensitized bacteria (10^8 organisms/ml), 0.1 ml of human complement, and 0.1 ml of lysozyme (1,000 μg/ml) were mixed and incubated at 37 C. After incubation for 18 hr, the inhibition of colony formation on agar plates was recorded.

Human complement was collected by venipuncture from a healthy person in this laboratory. After absorption of natural antibody against virulent strain 116-54 of *S. enteritidis*, the serum was filtered through a DA filter pad (Millipore Corp., Bedford, Mass.), divided into small volumes, and stored at −80 C. The titer of complement was 55 complement hemolyzing units per ml.

Lysozyme was a sample crystallized three times and purified from egg white and was purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

The monocytes of mice which had been treated with an immune RNA preparation demonstrated cellular immunity, inhibited intracellular multiplication of bacteria, and resisted cell degeneration caused by infection. Representative results are shown in Fig. 1 and 2.

When the normal monocytes were incubated in vitro with an immune RNA preparation extracted with phenol from immune monocytes, they also

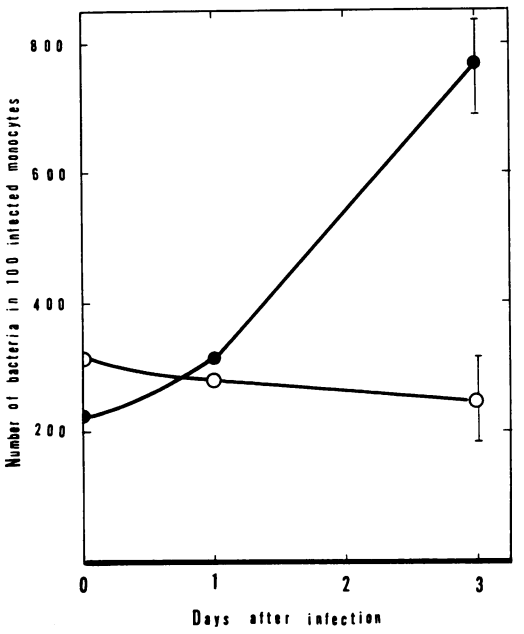

FIG. 1. *In vivo transfer of cellular immunity by an immune RNA preparation and inhibition of intracellular multiplication of Salmonella enteritidis 116-54. Symbols:* ○, *monocytes of mice which were treated with the RNA preparation extracted with phenol from the monocytes of mice hyperimmunized with live vaccine of S. enteritidis;* ●, *monocytes of mice which were treated with the RNA preparation extracted with phenol from normal mouse monocytes.*

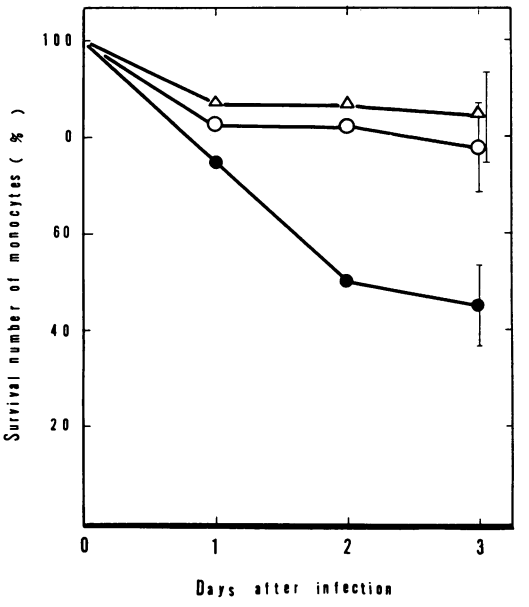

FIG. 2. *In vivo transfer of cellular immunity by an immune RNA preparation and resistance against cell degeneration caused by infection. See legend of Fig. 1 for circle symbols;* △, *normal monocytes without infection.*

acquired cellular immunity, inhibited intracellular multiplication of bacteria, and resisted cell degeneration caused by engulfment of bacteria (Fig. 3 and 4).

TABLE 1. *Formation of cellular antibody in the monocytes treated in vivo or in vitro with an immune RNA preparation*[a]

Expt no.	Monocytes	No. of monocytes per reaction mixture							
		8×10^7	4×10^7	2×10^7	1×10^7	0.5×10^7	0.25×10^7	0.12×10^7	None
1	Treated in vivo with immune RNA[b]	++	+	+	+	+	−	−	−
	Treated in vivo with normal RNA[c]	−	−	−	−	−	−	−	−
2	Treated in vitro with immune RNA[b]	++	+	+	+	+	−	−	−
	Treated in vitro with normal RNA[c]	±	−	−	−	−	−	−	−
Control	Immune RNA alone[d]	−	−	−	−	−	−	−	−
	Normal RNA alone[e]	±	−	−	−	−	−	−	−

[a] Monocytes in experiment 1 were collected from the peritoneal cavity of mice which were injected intraperitoneally with the phenol-extracted RNA preparations 5 days prior to collection. Monocytes in experiment 2 were treated in vitro for 3 days with RNA preparations. Monocytes were used for the source of antibody. IAHA titers of 0 to 1, 2, 3, and 4 were recorded as −, +, ++, and +++, respectively (18).

[b] RNA preparation extracted with phenol from immune monocytes.

[c] RNA preparation extracted with phenol from normal monocytes.

[d] Same as *a*, but monocytes were omitted.

[e] Same as *b*, but monocytes were omitted.

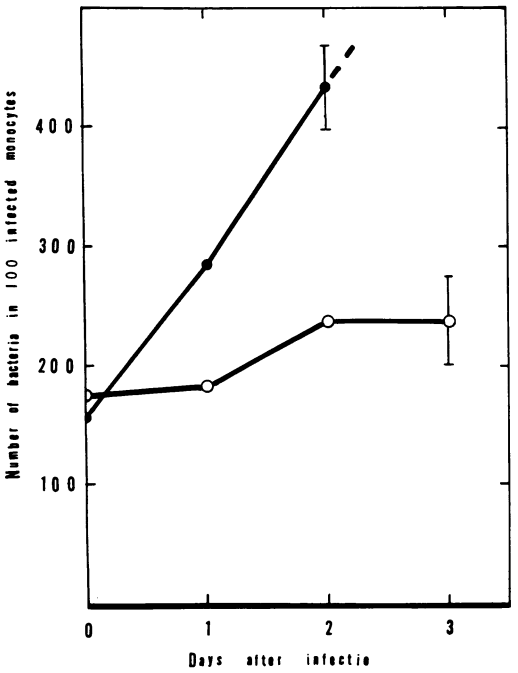

FIG. 3. *In vitro transfer of cellular immunity by an immune RNA preparation and inhibition of intracellular multiplication of Salmonella enteritidis 116-54. Symbols: ○, monocytes treated in vitro with the RNA preparation extracted with phenol from the immune monocytes; ●, monocytes treated in vitro with the RNA preparation extracted with phenol from normal monocytes.*

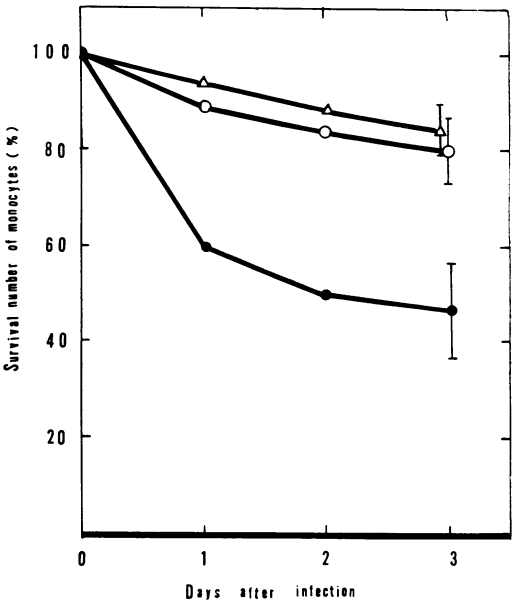

FIG. 4. *In vitro transfer of cellular immunity and resistance against cell degeneration caused by infection. See legend of Fig. 3 for circle symbols; △, normal monocytes without infection.*

Formation of cellular antibody in the monocytes of mice treated with an immune RNA preparation. The relation between the formation of cellular antibody and acquisition of cellular resistance after treatment with an immune RNA preparation was investigated. As shown in Table 1, the cellular antibody was detectable in the monocytes which were treated in vivo or in vitro with an immune RNA preparation extracted with phenol from immune monocytes. The controls, the monocytes which had been treated with the phenol-extracted RNA preparation from normal monocytes, did not contain antibody.

Inhibition of bacterial growth by the cellular antibody produced in monocytes treated in vivo or in vitro with an immune RNA preparation. The monocytes were treated in vitro or in vivo with an immune RNA preparation extracted with phenol from the monocytes of immunized mice. Antibody produced by these monocytes was transferred to live organisms of virulent strain 116–54. As shown in Table 2, the colony formation of bacteria was reduced to 1.1 to 2.0% of control, when the bacteria were treated with the monocytes of mice which had received an immune RNA preparation. Similarly, the monocytes which had been treated in vitro with an immune RNA preparation inhibited bacterial growth, and colony formation was reduced to 0.9 to 2.3% of control.

DISCUSSION

In 1961, a biological role of RNA in immune reactions was indicated by work in this laboratory (S. Mitsuhashi et al., Abstr. Meeting Japan. Bacteriol. Assoc. p. 19, 1961) and by Fishman (6). Since then, this subject has been studied by a number of other investigators (2–4, 7–15, 18, 39, 40, 44). Two divergent views on the role of RNA in antibody formation have developed. One considers the RNA functional per se (Saito and Kurashige, *unpublished data*), whereas the other places more emphasis upon the role of the antigen which is complexed with the RNA (2, 3, 13).

Up to now, it has not been clearly understood whether the functional RNA is RNA which carries the information for antibody formation or plays a role for antigenic information as a complex of RNA and antigen, or fragment thereof.

Monocytes have been reported to be associated with cellular immunity in infections of guinea pig with *Mycobacterium tuberculosis* (10, 19, 22, 41), of sheep with *Listeria monocytogenes* (20, 33), of rabbits and guinea pig with *Pasteurella tularensis* (42, 43), and of mice with salmonellae (5, 21).

TABLE 2. *Inhibition of bacterial growth by cellular antibody produced in monocytes treated in vivo or in vitro with an immune RNA preparation*[a]

Treatment of Ag	Inhibition of bacterial growth (%)	
	Expt 1	Expt 2
Ag	0	0
Ag + C′ + lysozyme	6.3	—[f]
Ag treated with the monocytes of mice which had received immune RNA[b] + C′ + lysozyme	98.9	98.0
Ag treated with the monocytes of mice which had received normal RNA[c] + C′ + lysozyme	8.0	0
Ag + monocytes treated in vitro with immune RNA[b] + C′ + lysozyme	99.1	97.7
Ag + monocytes treated in vitro with normal RNA[c] + C′ + lysozyme	4.5	3.7
Ag treated with immune RNA[d] + C′ + lysozyme	5.3	6.0
Ag treated with normal RNA[e] + C′ + lysozyme	4.0	5.8

[a] Antigen (Ag), 10^8 organisms of virulent strain 116-54 per ml; complement (C′), human complement. Ag was mixed with the monocytes treated in vivo or in vitro with the phenol-extracted RNA preparations as described in Materials and Methods, and cellular antibody was transferred to Ag.
[b–e] See the corresponding footnotes in Table 1.
[f] Not done.

It was concluded that host resistance in immunity by live organisms is primarily dependent on the acquired immunity of mononuclear phagocytes which have enhanced intracellular destructive capacities, although immune serum was needed for cellular immunity in the immune monocytes and in immune serum system in the infection of guinea pig with *M. tuberculosis* and of sheep with *L. monocytogenes* (10, 33).

The specificity and the cross immunity of cellular antibody in immune monocytes or of cellular antibody produced by the monocytes treated in vitro or in vivo with an immune RNA preparation will be described elsewhere.

The use of a homogeneous monocyte population in the present study has permitted an investigation of the possible role of this cell type in antibody formation. The finding that treatment of these cells with RNA from immune mice causes the appearance of cellular antibody and cellular immunity suggests that mononuclear phagocytes are capable of antibody formation (25, 26).

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