Experimental Salmonellosis

VI. In Vitro Transfer of Cellular Immunity of Mouse Mononuclear Phagocytes

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ABSTRACT

SAITO, KAZUKO (Gunma University, Maebashi, Japan), AND SUSUMU MITSUHASHI. Experimental salmonellosis. VI. In vitro transfer of cellular immunity of mouse mononuclear phagocytes. J. Bacteriol. 90:629–634. 1965.—A culture medium of the mononuclear phagocytes (monocytes) of mice immunized with live vaccine of Salmonella enteritidis contains the transfer agent (TA) of cellular immunity from immune to nonimmune monocytes. The TA is of ribonucleic acid nature, is nondialyzable through cellophane, and maintains its active state for 3 months in a frozen state (−10 C) and for 24 hr at 37 C.

In previous papers (Mitsuhashi, Sato, and Tanaka, 1961; Sato et al., 1962) it was reported that the mononuclear phagocytes (monocytes) of mice superimmunized with live vaccine of Salmonella enteritidis inhibited the intracellular multiplication of virulent S. enteritidis 116-54, and that they resisted cell degeneration caused by infection in the absence of an immune serum in the tissue-culture medium. Also, it was found that the supernatant fluid of a culture of immune monocytes (designated as SFC) is capable of conferring cellular immunity to nonimmune monocytes. The agent present in the culture medium thus obtained was referred to as the transfer agent (TA) of cellular immunity (Mitsuhashi and Saito, 1962; Mitsuhashi, Sato, and Tanaka, 1964). This paper presents the results obtained from studies made on the properties of TA.

MATERIALS AND METHODS

Experimental animals, immunization procedure, and microorganisms used were described previously (Mitsuhashi et al., 1961). Heat-killed bacteria were prepared for use as antigen of dead vaccine.

The collection of mononuclear phagocytes, the phagocytic system, and tissue culture were as described previously (Mitsuhashi et al., 1961).

Preparation of SFC. Mononuclear phagocytes were obtained from the abdominal cavity of normal or immune mice which had been intraperitoneally administered 1 ml of glycogen solution (0.01 mg/ml in physiological saline) 4 to 5 days prior to their collection. Cells were obtained from the abdominal cavity with a syringe, and they were washed twice by centrifugation at 335 X g for 3 min in the cold. The cells were then suspended in a cold culture medium of 30% horse serum and 70% Hanks solution, containing penicillin (25 units per ml). The suspension was adjusted to contain 1,500 cells per cubic millimeter. A 10-ml amount of cell suspension thus obtained was placed in a large tissue-culture bottle and incubated at 37 C. The cells adhered to the bottom of the tissue-culture bottles. After 6 hr of incubation, the supernatant fluid was removed and replaced with a fresh culture medium containing heat-killed cells of S. enteritidis 116-54 (10^-4 mg/ml; corresponding to 3 X 10^4 cells per milliliter). After 24 hr of incubation at 37 C, the culture medium was centrifuged at 335 X g for 5 min and filtered through Millipore HA filter pads.

In vitro transfer of cellular immunity. Monocytes obtained from the abdominal cavity of normal mice were suspended in culture medium; 2 ml of cell suspension (1,000 cells per cubic millimeter) were placed in a culture chamber provided with cover glasses, and were incubated at 37 C. Monocytes adhered to the cover glasses. The supernatant fluid was removed after 6 hr of incubation and replaced with fresh culture medium containing penicillin (25 units per ml) and 50% SFC, obtained from normal or immunized mice. The culture medium containing the SFC was changed daily. After 72 hr of incubation, the supernatant fluid was removed, and the monocytes were infected with a virulent strain (116-54) of S. enteritidis suspended in fresh culture medium (without antibiotics) containing 5% normal mouse serum. The multiplicity of infection of bacteria to monocytes was 0:1 to 0:1. After 60 min of incubation at 37 C, the supernatant fluid containing the bacteria was discarded and replaced with a fresh culture medium containing penicillin (10 units per ml) and streptomycin (10 μg/ml) to inhibit the extracellu-
lar growth of bacteria in tissue-culture bottles. This concentration of antibiotics did not inhibit the intracellular growth of bacteria in normal mouse monocytes. The medium was changed daily. The cover glasses, which monocytes adhered, were removed from the culture bottle at appropriate intervals of incubation after infection (0, 6, 12, 24, and 72 hr), dried in air, fixed in methanol, and stained with Giemsa solution.

The number of monocytes, infected monocytes, and intracellular bacteria in monocytes was determined microscopically. The phagocytic index was calculated by the following ratio: (number of monocyte which ingested bacteria)/(total number of monocytes examined). The total number of monocytes which adhered to the cover glass was expressed as a mean of three counts after counting the monocytes in five microscopic fields at a magnification of 200. The standard deviation of the number of cell numbers on each cover glass in the same culture bottle was calculated as about 10%, and the deviation of phagocytic index was controlled so as to remain below 5%.

Treatment of TA with nucleases. Ribonuclease (crystalline, Worthington Biochemical Corp., Freehold, N. J.) and deoxyribonuclease (crystalline, Sigma Chemical Co., St. Louis, Mo.) were dissolved in Hanks’ solution of pH 7.0. The TA was treated with nucleases (50 μg/ml) at 37°C. As an untreated control, the TA was incubated at 37°C without addition of nucleases. After 12 hr of incubation, the transfer of cellular immunity by TA, treated or untreated with nucleases, was examined as described above by the addition of TA to a concentration of 50%. The nucleases had no harmful effect on monocytes in the medium containing 50% of the original nuclease solution after 12 hr of incubation at 37°C.

Stability of TA on standing. The pooled TA was dispensed in equal amounts into small test tubes and stored at 57°C or in a frozen state (−10°C). At appropriate intervals, the activity of TA was determined as described above.

Permeability of TA. A 10-ml TA solution was dialyzed in a cellophane bag (Visking Corp., Chicago, Ill.) against 1 liter of Hanks’ solution in the cold, with continuous rotation by means of a magnetic stirrer. The outer solution was replaced with a fresh Hanks’ solution after 4 hr of dialysis. After 8 hr of dialysis, the activity of TA inside the bag was examined by adding a concentration corresponding to 50% of the original TA.

Fractionation by centrifugation. The pooled TA solution was centrifuged at 100,000 X g for 2 hr in a refrigerated centrifuge, model 55 p (Hitachi Co.). Thereafter, the activities of the supernatant fluid and of the precipitate, dissolved in Hanks’ solution to the original volume, were measured.

RESULTS

Transfer of cellular immunity through SFC. The SFC was obtained from the cultivation of monocytes from normal or immunized mice. The transfer of cellular immunity to normal monocytes through SFC was carried out as described in Materials and Methods. As shown in Fig. 1 and 2, the SFC obtained from the monocytes of mice immunized with live vaccine of S. enteritidis conferred cellular immunity to normal monocytes. The treated monocytes inhibited the intracellular multiplication of virulent S. enteritidis 116-54 and resisted the cell degeneration caused by the engulfment of bacteria. In contrast, the SFC obtained from normal monocytes or from the monocytes of mice immunized with dead vaccine was unable to confer cellular resistance, and the treated monocytes were destroyed after infection. Hereafter, the agent which is present in the SFC of immune monocytes is referred to as the TA of cellular immunity.

Effect of dilution on the activity of TA. The original TA contained in the SFC of immune monocytes was diluted with fresh culture medium. The nonimmune monocytes were incubated in a culture medium containing 50% of the original TA or of each dilution. After 72 hr of incubation at 37°C, the medium was replaced with fresh culture medium, and the resistance of cells was deter-

![Graph](http://jb.asm.org/diag1.jpg)  
**Fig. 1.** Inhibition of intracellular multiplication of Salmonella enteritidis by treatment with TA. Symbols: , SFC from normal mice; , SFC from mice immunized with live vaccine; , SFC from mice immunized with live vaccine of S. enteritidis. Each point indicates the mean of three experiments.
mined by infecting them with *S. enteritidis* 116-54. As shown in Table 1, the monocytes pretreated with 1:2 or 1:4 dilution of the original TA acquired cellular immunity, and they resisted cell degeneration caused by engulfment of bacteria; those pretreated with a 1:8 dilution of original TA did not acquire immunity or resist degeneration.

**Effect of nucleases upon the activity of TA.** The TA was obtained from the monocytes of mice hyperimmunized with live vaccine of *S. enteritidis* and treated with nucleases. Nonimmune monocytes were incubated with 50% (1:2 dilution) of the original TA or 50% of the TA treated with nucleases, and the immune transfer was examined. As shown in Fig. 3 and 4, the number of monocytes incubated in the TA pretreated with ribonuclease decreased rapidly after infection, and the number of bacteria in the infected monocytes increased, causing destruction of the cell within 1 day after infection. For the control experiment, the effect of ribonuclease on the normal monocytes was examined. The same concentration of ribonuclease as in the treatment of TA, after incubation at 37°C for 12 hr, produced no

<table>
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<tr>
<th>Dilution of TA</th>
<th>Transfer of cellular immunity</th>
<th>Number of bacteria in 100 infected monocytes</th>
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<tr>
<td></td>
<td>Per cent survival of monocytes after infection</td>
<td>Per cent survival of monocytes after infection</td>
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<tr>
<td></td>
<td>0 hr</td>
<td>12 hr</td>
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<tr>
<td>1:2</td>
<td>100%</td>
<td>96%</td>
</tr>
<tr>
<td>1:4</td>
<td>100%</td>
<td>96%</td>
</tr>
<tr>
<td>1:8</td>
<td>100%</td>
<td>91%</td>
</tr>
<tr>
<td>Without TA</td>
<td>100%</td>
<td>82%</td>
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*The number of monocytes is expressed as the percentage of the initial number. The per cent survival of monocytes was obtained from counts of five microscopic fields in each experiment.*
harmful effect on the cell. In contrast, the original TA and also the TA pretreated with deoxyribonuclease were able to confer cellular immunity to nonimmune monocytes, and the cells inhibited the intracellular multiplication of bacteria; there was no noticeable decrease in cell number after infection.

Effect of storage on the activity of TA. The TA obtained from the immune monocytes was maintained at 37°C or in a frozen state (−10°C). At appropriate intervals of storage, the activity of TA was examined. Even after 3 months in a frozen state or after 1 day at 37°C, the activity of TA was still present, whereas no activity was observed after 5 months in a frozen state (Fig. 5).

The TA of SFC obtained from the monocytes of mice hyperimmunized with live vaccine of S. enteritidis was placed in a cellophane bag and dialyzed in the cold with continuous rotation against Hanks' solution. After 1 day of dialysis, the activity was present, indicating that the TA is nondialyzable through the cellophane.

Fractionation of TA by centrifugation. The TA was purified by fractional centrifugation. The TA

![Figure 4](http://jb.asm.org/)

**Fig. 4.** Loss of activity of TA by treatment with ribonuclease and decrease in cell number by infection. Symbols as in Fig. 3.

![Figure 5](http://jb.asm.org/)

**Fig. 5.** Stability of TA for preservation. TA was maintained in a frozen state. The numbers (1, 3, 5, and 6) in the graph show the length of storage in months.

![Figure 6](http://jb.asm.org/)

**Fig. 6.** Loss of activity of TA by centrifugation at 100,000 × g and recovery of activity by mixing supernatant fluid with sediment. Symbols: ○, sediment plus supernatant fluid of TA; ▲, sediment of TA; ◊, supernatant fluid of TA; ●, without TA; ●, original TA before centrifugation.
in SFC of immune monocytes was centrifuged at 100,000 × g for 2 hr. The precipitate was suspended in a volume of the same medium employed in the original TA solution. The transfer activities of the supernatant fluid and precipitate thus obtained were determined by adding one-fourth of the original TA solution to the culture medium of nonimmune monocytes. As shown in Fig. 6, the activity of both fractions obtained by centrifugation was decreased in comparison with that of the original TA. However, the activity was fully restored when the precipitate was combined with the supernatant solution. When one-half of the original TA was added to the culture medium, the transfer activity of the precipitate was almost the same as with one-fourth of the original TA solution. From these results, it was concluded that TA was precipitated by centrifugation at 100,000 × g for 2 hr, whereas the transfer activity of the supernatant fluid was restored by adding increasing amounts.

**DISCUSSION**

Passive transfer of acquired immunity or hypersensitivity from the donors to normal recipient animals offers a powerful tool for the elucidation of cellular events by which immunity or hypersensitivity is established.

By the transfer of cells from BCG-vaccinated donors to normal recipients, some degree of protection against challenge infection with tubercle bacilli was achieved in guinea pigs and mice (Suter, 1961).

It was also found that passive transfer of the peritoneal exudate cells from the immune donor mice, immunized intraperitoneally with the H37Ra strains of *Mycobacterium tuberculosis* var. *hominis*, protected normal recipient mice from infection with massive doses of virulent tubercle bacilli (Millman, 1962).

Fong, Schneider, and Elberg (1957) demonstrated that immune histiocytes, obtained from the abdominal cavity of BCG-vaccinated rabbits, resisted cell degeneration caused by parasitization with the virulent H37Rv strain in the presence of heterologous antisera (anti-*Salmonella* and anti-ovalbumin) as well as of homologous antiserum (anti-BCG) in the tissue-culture medium. It was further demonstrated that the lysates of immune histiocytes, as well as the original immune histiocytes from BCG-vaccinated rabbits, were able to transfer cellular resistance and that the histiocytes from recipient animals withstood cell degeneration caused by the engulfment of virulent H37Rv strains at the end of the transfer series (Fong, Chin, and Elberg, 1962). This active principle which participated in the induction of cellular resistance was an active ribosomal ribonucleic acid (RNA) of immune histiocytes; thus, this material was inactivated by treatment with ribonuclease and not with deoxyribonuclease or trypsin (Fong et al., 1963).

In the infection of mice with *S. enteritidis*, it was reported that cultured peritoneal macrophages of mice, which had been injected intravenously with intact macrophages of mice immunized with live vaccine, inhibited the intracellular multiplication of virulent *S. enteritidis*. This inhibition, however, was inferior to that exhibited by the peritoneal macrophages obtained from immune donors. From their experiment with P32-labeled macrophages, Saito et al. (1962) concluded that the inhibitory action of the recipient macrophages against intracellular multiplication was due to the capacity of the recipient's own macrophages. From the same laboratory, it was also reported that extracts of immune histiocytes were unable to transfer cellular immunity and that both the injection of intact viable cells (living contaminated microorganisms used as live vaccine) and the growth of bacteria in the recipients were indispensable for the effective transfer of cellular immunity (Akiyama, 1962). The fundamental problem which exists in transfer of cellular immunity in experimental salmonellosis is still open for future study.

Previous reports and the results described herein indicate that, in the transfer of either immunity or hypersensitivity, an active extract from immune or sensitive cells is RNA in nature (Fong et al., 1963; Jankovic and Dorak, 1962; Mannick and Egdahl 1964; Sato, Kato, and Mitsushashi, 1964), but the nature of transfer factor remains conjectural. It is necessary to determine which component of cell materials, i.e., ribosome or some other cellular component, is the active principle containing the RNA.

Fox, Yoon, and Mead (1962) found a structural modification of a specific protein in *Drosophila melanogaster* in the absence of genetic material responsible for its formation. This finding suggests the existence of an enzymatic system for the synthesis of RNA with preformed RNA as a primer. It has also been reported that the ribonucleoproteins and RNA may cause functional and morphological modifications in vertebrate systems, e.g., alterations in homograft tolerance and antibody formation (Hess, Corrigan, and Hodak, 1961; Niu, Cordova, and Niu, 1961; Ashley et al., 1960; Hrubesova, Askonas, and Humphrey, 1959).

In a previous paper (Mitsushashi, 1964), it was reported that cell-bound antibody was detected in the abdominal monocytes of mice hyperim-
munized with live vaccine of *S. enteritidis*. This antibody inhibited the growth of virulent *S. enteritidis* either on the plate or in normal mouse monocytes in the presence of complement and lysozyme (Kurashige et al., 1964). The relationship which exists between this cell-bound antibody in immune monocytes and the TA in SFC or ribosomal fraction of immune monocytes will be described elsewhere.

**Acknowledgments**

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**Literature Cited**


