Transfer Factor from BCG-Sensitized Mice

KENJI SASAKI, FUJIO SUZUKI and NAKAO ISHIDA

Department of Bacteriology, Tohoku University School of Medicine, Sendai 980

SASAKI, K., SUZUKI, F. and ISHIDA, N. Transfer Factor from BCG-Sensitized Mice. Tohoku J. exp. Med. 1980, 131 (3) 271-283 — A mouse model was established for the study of transfer factor (TF). TF was extracted from the spleens of sensitized mice and examined for activity by the footpad test, which showed that mouse and human TF possessed similar properties. Parenteral administration of TF imparted to unsensitized mice immunologically specific, delayed type hypersensitivity within 24 hr. The magnitude of the response the recipients showed was proportional to the dose of TF. TF activity was relatively heat-stable, detectable in a fraction of molecular weight of ca 1,000 and apparently contained nucleic acids. Nude mice responded negatively to TF but following administration of viable naive spleen cells a positive response was observed, which suggests that the thymus plays an important role in the expression of TF phenomenon and that the target of TF is the T cells. —— transfer factor; animal model; BCG-sensitized mice; delayed type hypersensitivity

After a long latent period since its discovery by Lawrence in 1955 (Lawrence 1955), transfer factor (TF) has recently moved into the limelight as the antigen-specific initiator of cell-mediated immunity and has been widely applied with promising results to the treatment of a variety of immunodeficiency diseases (Levin et al. 1970; Spitler et al. 1972; Ammann et al. 1974; Ballow and Good 1975), intracellular infections (Schulkind et al. 1972; Graybill et al. 1973; Wolf et al. 1978), and malignancies (Brandes et al. 1971; Oettgen et al. 1974). However, its character or mode of action is little understood because TF phenomenon, though easily demonstrated in humans in vivo, has been detected with difficulty in experimental animals (Lawrence 1969): even the very promising results obtained with DNCB-sensitized guinea pigs have not been reproducible (Dressler and Potter 1975). The clinical application of TF is, therefore, dependent on nothing but empiricism and the establishment of an animal model system is needed before the clarification of the properties and mechanism of the action of TF can be made.

We have investigated several combinations of animals and antigens in order to establish an animal model system and have discovered TF activity in a fraction extracted from the spleens of sensitized mice. This paper reports the properties and possible mechanism of action of this material.
MATERIALS AND METHODS

Preparation of TF. Male and female ddI mice, over 6 weeks old and weighing 20 g or more, which were propagated at Tohoku University were used throughout this study as TF donors and recipients. The animals were sensitized by a single intravenous injection of $5 \times 10^4$ to $10^5$ BCG (Koseikai BCG Laboratory, Sendai, Japan) or $2 \times 10^5$ viable *Candida albicans* suspended in 0.1 ml of phosphate-buffered saline (PBS) (pH 7.2-7.4). Three weeks later, the spleens were removed and disrupted with an omnimixer at 16,000 rpm for 60 min in ice water. This was followed by 10 cycles of freezing and thawing and then centrifugation at 25,000 rpm for 120 min at 4°C. The supernatant was cleared of substances with molecular weights of 10,000 or more by ultrafiltration through an Amicon Diaflo PM-10 filter. The filtrate was lyophilized and redissolved in PBS so that the extract from 5 spleens was contained in 1 ml of the solution, and stored at -20°C until used.

Assessment of TF activity. The activity of TF was assayed by the footpad test: an eliciting dose of 30-50 μg of PPD (Parke Davis), $8 \times 10^4$ to $10^5$ BCG, or $5 \times 10^4$ heat-killed *Candida albicans* in 0.1 ml of PBS was injected into the subcutaneous tissues of the plantar surfaces of the hind footpads of ddI or nude mice which had been given TF 24 hr before. The degree of swelling was measured 24 hr later with a micrometer with circular plane surfaces reading to 0.001 mm (Mitsutoyo Seisakusho Co., Ltd., Tokyo, Japan).

The reaction was expressed as the difference in thickness between the challenged and unchallenged footpads.

Gel filtration of TF. BCG-specific TF powder prepared from 150 spleens was redissolved in distilled water and centrifuged at 15,000 rpm for 120 min at 4°C. The supernatant was applied in a sample volume of 5 ml to a 2 x 100 cm Sephadex G-25 column and eluted with distilled water. Five ml fractions were collected and the optical density of each was measured at 260 and 280 nm.

Fraction III obtained by Sephadex G-25 gel filtration of crude TF was lyophilized to a white powder. Two hundred ng of the powder was redissolved in distilled water, further fractionated on a 0.7 x 48 cm Sephadex G-15 column and eluted with distilled water. Two-ml fractions were collected and analyzed for absorbance at 260 and 280 nm.

RESULTS

Response to TF of normal mice

Twenty-four hr after i.p. injection with TF prepared from the spleens of 5 BCG-sensitized mice, 7 normal ddI mice were footpad tested with BCG. Eight untreated and 3 BCG-sensitized mice served as negative and positive controls, respectively. In untreated mice, the challenged footpads swelled by 0.94±0.12 mm (mean value±s.d.) apparently due to a nonspecific inflammatory reaction to BCG, whereas, in BCG-sensitized and TF-treated mice, they increased by 1.93±0.28 and 2.37±0.26 mm, respectively (Fig. 1). Thus TF activity was confirmed ($p<0.001$, by t-test).

Response to naive TF of normal mice

Three groups each comprising 5 normal mice were given i.p. different doses of spleen extract from unsensitized mice (termed naive TF), which were prepared by the same procedure as TF, and were footpad tested, as described above. Six untreated mice and 2 mice given TF prepared from the spleens of 2.5 BCG-sensitized mice served as negative and positive controls, respectively. Though BCG-TF
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Fig. 1. Response to TF of normal mice as measured by the footpad test. TF-treated mice were footpad tested with BCG. BCG-sensitized and untreated mice served as positive and negative controls, respectively. Two circles connected with a line represent the respective thicknesses of challenged and unchallenged footpads of individual mice. R, right footpad, untreated; L, left footpad, challenged with BCG.

<table>
<thead>
<tr>
<th>Mice given</th>
<th>Increase in footpad thickness (mean±s.d., mm)</th>
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</thead>
<tbody>
<tr>
<td>BCG-TF 2.5</td>
<td>1.77±0.09</td>
</tr>
<tr>
<td>No TF</td>
<td>1.25±0.15</td>
</tr>
<tr>
<td>Naive TF 2.5</td>
<td>1.41±0.18</td>
</tr>
<tr>
<td>Naive TF 5.0</td>
<td>1.36±0.13</td>
</tr>
<tr>
<td>Naive TF 10.0</td>
<td>1.34±0.07</td>
</tr>
</tbody>
</table>

exerted an effect \(p<0.001\), naive TF was found to be inactive (no significant difference was observed at the 20\% level) (Table 1). TF activity was, therefore, detectable only in the spleen extracts from the sensitized animals.

Dose response to TF

The relationship was examined between the dose of TF and the magnitude of the sensitivity transferred to the recipients. Four groups of 2–7 normal mice received different doses of TF from BCG-sensitized mice and were footpad tested, as described above. Three BCG-sensitized and 8 normal mice served as positive and negative controls, respectively. Fig. 2 shows the more TF given, the stronger the sensitivity transferred. TF prepared from 3 spleens gave the same degree of sensitivity as was observed in the BCG-sensitized mice (no difference was observed at the 50\% level) and TF prepared from 5 or more spleens conferred stronger sensitivity \(p<0.005\).

Heat-stability of TF

Aliquots of TF prepared from BCG-sensitized mice were treated respectively at 4°C for 72 hr, at 37°C for 60 min, and at 56°C and 75°C for 30 min. Twelve mice
Fig. 2. Dose response to TF. Mice injected with different doses of TF were footpad tested with BCG. BCG-sensitized and untreated mice served as positive and negative controls, respectively. Each circle and horizontal bar represent the mean value and s.d. of each group, respectively. The dose of TF is expressed in terms of the number of spleens from which it was prepared. The shaded area represents the average value±s.d. of the positive control.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Increase in footpad thickness (mean±s.d., mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>0.94±0.12</td>
</tr>
<tr>
<td>Positive control</td>
<td>1.92±0.24</td>
</tr>
<tr>
<td>4°C for 72 hr</td>
<td>1.73±0.39</td>
</tr>
<tr>
<td>37°C for 60 min</td>
<td>2.03±0.14</td>
</tr>
<tr>
<td>56°C for 30 min</td>
<td>2.16±0.05</td>
</tr>
<tr>
<td>75°C for 30 min</td>
<td>1.26±0.28</td>
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It was observed that TF activity, though partially inhibited by treatment at 75°C for 30 min, \(p<0.025\), was unaffected by any other treatment at the 50% level (Table 2).

**Antigen-specificity of TF**

Ten mice were given i.p. TF prepared from the spleens of 5 Candida albicans-sensitized mice. Five of the former mice were footpad tested with BCG and the others were tested with heat-killed Candida albicans 24 hr later. At the same time,
another 10 mice were injected i.p. with TF prepared from the spleens of 5 BCG-sensitized mice and were divided into two groups and tested with either antigen, as mentioned above.

Table 3 shows that the mice given Candida-TF responded to an eliciting injection of Candida, while those receiving BCG-TF showed only little reaction to that antigen \( (p<0.025) \). In the same manner the recipients of BCG-TF mounted a significantly greater response to a challenging injection of BCG than those that received Candida-TF \( (p<0.001) \). TF was, therefore, shown to confer an antigen-specific immunological memory upon a recipient.

**Response of nude mice to TF**

After receiving an i.p. injection of BCG-specific TF prepared from 5 spleens, 2 nude mice were footpad tested with 30 µg of PPD 24 hr later. Two similarly treated ddI mice and 2 untreated nude mice served as positive and negative controls, respectively.

Fig. 3 shows that, though the TF-treated ddI mice showed a significant response to TF, the treated nude mice showed no response at all. This result

<table>
<thead>
<tr>
<th>Mice</th>
<th>Increase in footpad thickness (mean±S.D., mm)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Challenged with</td>
<td>Given</td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>C. albicans-TF</td>
<td>0.32±0.13</td>
</tr>
<tr>
<td></td>
<td>BCG-TF</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>GCB</td>
<td>BCG-TF</td>
<td>1.66±0.11</td>
</tr>
<tr>
<td></td>
<td>C. albicans-TF</td>
<td>1.18±0.10</td>
</tr>
</tbody>
</table>

Fig. 3. Response of nude mice to TF. TF-treated nude mice were footpad tested with PPD. TF-treated and untreated ddI mice served as positive and negative controls, respectively. Columns and horizontal bars represent the mean values and s.d., respectively.
suggests that the thymus plays an important role in the expression of TF-conferred hypersensitivity.

Reconstitution of responsiveness of nude mice to TF

Three nude mice were injected i.p. with $2 \times 10^8$ viable spleen cells collected from normal ddI mice. Ten min later two of the recipients were given BCG-specific TF prepared from 5 spleens and were footpad tested with 50 $\mu$g of PPD 24 hr later. Two nude mice were given the same number of viable spleen cells from ddI mice which had received a similar amount of BCG-specific TF 24 hr previously and served as positive controls. Another 2 nude mice were treated with BCG-specific TF alone to serve as negative controls.

Table 4 shows that the animals given both naive splenocytes and TF responded to the challenge as well as those receiving splenocytes from TF-treated mice and the difference between the two was not statistically significant ($p > 0.10$). The responsiveness of nude mice to TF proved, therefore, to be reconstituted by the introduction of viable splenocytes.

<table>
<thead>
<tr>
<th>Mice given</th>
<th>Increase in footpad thickness (mean±S.D., mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF</td>
<td>0.00</td>
</tr>
<tr>
<td>Naive splenocytes</td>
<td>0.00</td>
</tr>
<tr>
<td>Naive splenocytes+TF</td>
<td>0.23±0.03</td>
</tr>
<tr>
<td>Splenocytes from TF-treated mice</td>
<td>0.28±0.10</td>
</tr>
</tbody>
</table>

Chromatography of TF on Sephadex G-25

Fig. 4 shows an elution profile of BCG-specific TF prepared from 150 spleens obtained by gel filtration on a 2×100 cm Sephadex G-25 column. The sample was divided into 4 fractions on the basis of absorbancy at 260 nm. The eluate with the highest optical density in each fraction was administered i.p. to 5 ddI mice which were then tested for reactivity to BCG.

Table 5 shows that the most potent activity resided in fraction III ($p < 0.001$), which eluted at $2.9 \times V_0$. The fraction was shown to induce the sensitized state at the level of 3.5 mg/kg of body weight or 70 $\mu$g/mouse which is comparable to that induced by BCG. Crude TF reacted at the level of 1050 mg/kg. The most active fraction thus appeared to be 300 times as active as crude TF.

The active fraction recovered was 90% by activity and 0.3% by weight of the sample applied to the column.

Rechromatography of the active fraction on Sephadex G-15

Fractionation of 200 ng of the powder from the active fraction on Sephadex G-15 resulted in 2 sharp peaks, as shown in Fig. 5. Three ddI mice were each given one-third of the first fraction and another 3 received one-third of the second
Fig. 4. Chromatography of TF on a Sephadex G-25 column. TF prepared from 150 spleens was redissolved in 5 ml of distilled water, applied to a 2×100 cm Sephadex G-25 column, and eluted with distilled water. The eluate was collected in consecutive 5 ml fractions and measured for absorbance at 260 (○―○) and 280 nm (●―●). The shaded area represents the most active fraction.

**Table 5. Activity and yield of each fraction obtained by gel filtration on Sephadex G-25**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Yield (mg)</th>
<th>Increase in footpad thickness (mean±s.d., mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude TF</td>
<td>1000</td>
<td>--</td>
</tr>
<tr>
<td>I</td>
<td>137</td>
<td>1.47±0.10</td>
</tr>
<tr>
<td>II</td>
<td>560</td>
<td>1.27±0.08</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>1.34±0.21</td>
</tr>
<tr>
<td>IV</td>
<td>4</td>
<td>1.33±0.21</td>
</tr>
<tr>
<td>Control</td>
<td>--</td>
<td>0.98±0.13</td>
</tr>
</tbody>
</table>

**Table 6. Comparison of activity of each fraction obtained by gel filtration on Sephadex G-15**

<table>
<thead>
<tr>
<th>Mice given</th>
<th>Increase in footpad thickness (mean±s.d., mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I</td>
<td>2.37±0.07(p&lt;0.001)</td>
</tr>
<tr>
<td>Fraction II</td>
<td>1.96±0.04</td>
</tr>
<tr>
<td>None</td>
<td>1.98±0.04</td>
</tr>
</tbody>
</table>

fraction. Five untreated mice served as negative controls. They were then footpad tested with BCG. Table 6 shows that the activity was detected only in the first fraction (p<0.001), which was eluted just after the void volume, while the second fraction showed no statistically significant activity as compared with the control.
Fig. 5. Rechromatography of Fraction III on a Sephadex G-15 column. Fraction III was lyophilized to a white powder. Two hundred ng of the powder was redissolved in distilled water, placed on a 0.7 x 48 cm Sephadex G-15 column, and eluted with distilled water. Two-ml samples were collected and the elution profiles were monitored at 260 (○—○) and 280 nm (●—●). The shaded area represents the active fraction.

Fig. 6. UV spectrum of the active principle. The active principle dissolved in distilled water was measured for ultraviolet absorbancy.

UV spectrum of the active principle

Fig. 6 shows the UV spectrum of the active principle obtained by gel filtration on Sephadex G-15. It was shown to have a maximum absorbancy at 249 nm and to have an $OD_{260}/OD_{280}$ ratio of 9:1. In addition it was found to be orcinol positive. It was, therefore, considered to be a substance containing nucleic acids.
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DISCUSSION

Chase (1945) demonstrated that specific delayed type hypersensitivity was transferred to unsensitized guinea pigs by the injection of peritoneal exudative leukocytes from sensitized guinea pigs, thus achieving a distinct separation of the category of immune responses initiated by cells from that of those mediated by immunoglobulins. Reproducing the phenomenon in humans with viable blood leukocytes (Lawrence 1949), Lawrence (1955) observed later that leukocyte extracts were as effective as viable cells. Such leukocyte extracts, viz., TF, as the name indicates, is thought of as a substance that transfers a specific memory of cell-mediated immunity from a sensitive donor to a naive recipient.

However, TF fraction has recently been shown to have many other activities, such as nonspecific conversion of lymphocytes in vitro (Müller et al. 1977), augmentation of lymphocyte transformation responses to PPD (Hamblin et al. 1976a) and to PHA in vitro (Hamblin et al. 1976b), chemotactic activity in vitro (Gallin and Kirkpatrick 1974), nonspecific augmentation of thymidine incorporation of an antigen-specific precommitted clone of lymphocytes (Cohen et al. 1976), stimulatory or inhibitory effects on lymphocyte transformation in vitro (Burger et al. 1976a), and both specific and nonspecific augmentation of lymphocyte transformation in vitro (Burger et al. 1976b; Littman et al. 1977). This is partly because TF is not a single substance but a mixture of chemically heterogeneous dialyzable molecules apparently possessing different biological effects. Some of these activities were not detected in the more purified fraction having classical TF activity (Burger et al. 1976; Littman et al. 1977) and augmentation of $^3$H-thymidine incorporation by lymphocytes in vitro in the presence of dialyzable TF (TFd) is not a measure of TF activity (Littman et al. 1977). The relationship between other activities in vitro and the classical TF activity in vivo remains to be clarified (Gallin and Kirkpatrick 1974; Burger et al. 1976a, b; Cohen et al. 1976; Hamblin et al. 1976a, b; Andron and Ascher 1978).

We regard the classical TF activity as the marker of activity TF possesses. We suspect that TF is not an artifact but a physiological substance with a teleological raison d’être endowed by Nature: it exercises its activity in the intercellular milieu in an individual. As the cellular transfer of delayed type hypersensitivity can be accomplished in rodents, there is no room for doubt that Nature has also bestowed such a substance upon vertebrates other than humans. The misfortunes that have dogged the animal experimental systems previously described cannot be due to the nonexistence of TF in nonhuman vertebrates.

On such a premise, we investigated several combinations of animals and antigens to find an animal model and finally succeeded in showing TF-like activity in a fraction extracted from the spleens of BCG-sensitized mice (Fig. 1). This fraction was identified as possessing TF on the basis of the following characteristics: the fraction from BCG-sensitized mice imparted BCG-sensitivity to naive recipients within 24 hr, whereas the same fraction prepared from unsensitized mice did not
The active fraction transferred antigen-specific delayed type hypersensitivity to recipients (Table 3).

We found the TF activity to be relatively heat-stable (Table 2). Gel filtration of the substance on Sephadex G-25 and G-15 columns and the UV spectrum showed it to consist of a principal component of ca 1,000 daltons molecular weight, apparently containing nucleic acids (Figs. 4, 5 and 6).

TF in human is generally considered to be an extract from educated lymphocytes made up of a peptide-RNA nucleotide complex of less than 10,000 molecular weight, which transmits to a naive recipient an immunologically specific, long-lasting memory of cell-mediated immunity (Kirkpatrick and Rifkind 1974). Our results, as described above, are in general accordance with this consensus.

Many investigators have shown that gel filtration of human TFd on Sephadex G-25 termed TFc or TFg produces a biologically active fraction with a characteristic effluent volume and high 260 nm/280 nm ratio (Zuckerman et al. 1974; O’Dorisio et al. 1976; Tomar et al. 1976). Such a fraction was considered to contain hypoxanthine as a major component (O’Dorisio et al. 1976; Tomar et al. 1976). This was later shown not to be responsible for the TF activity in vivo (Kirkpatrick et al. 1976). In addition, hypoxanthine was found to separate from the active subfraction of TFg designated as Tx which does not have a high 260 nm/280 nm (Wilson et al. 1977). The precise character of Tx remains to be elucidated.

We demonstrated that mouse TF also has a characteristically high 260 nm/280 nm ratio which elutes just before the total bed volume on Sephadex G-25 and just after the void volume on Sephadex G-15. UV absorbance spectroscopy showed that mouse TF has a maximum absorbance at 249 nm at neutral pH, while TFg and hypoxanthine were shown to have their maximum absorption at 251 nm (Wilson et al. 1977). Also mouse TF was found to be orcinol positive. These data suggested that mouse TF was very similar to human TFg or TFc. Further purification of mouse TF is necessary. As it is the critical lack of an experimental animal model that has hampered the study of TF, the experimental system we have established will make a contribution to the further investigation of TF.

The consensus on human TF led us to a hypothesis that TF acts upon thymus-derived cells to trigger their differentiation, which is completed within 24 hr and, therefore, requires no cell division. To test the validity of this hypothesis, we examined whether TF caused conversion in nude, congenitally athymic mice, and observed that they did not respond to TF administration. In accord with our finding, Lawlor et al. (1974) reported that 3 patients with a probable diagnosis of Nezelof syndrome did not respond to TF administration either immunologically or clinically. In addition, Fakhmann et al. (1974) observed the lack of effect of TF in 4 children with thymic dysplasia with normal Ig synthesis and allegedly attributed the observation to the absence of thymus-influenced lymphocytes in these patients.

Further, we demonstrated that the unresponsiveness to TF in nude mice was abolished by the introduction of viable spleen cells. Taken together, these results,
though preliminary, strongly support our hypothesis and suggest that the thymus plays a key role in the expression of the TF phenomenon and that the target of TF is the T cells. Arala-Chaves et al. (1976) drew the same conclusion from in vitro studies.

We observed a dose response effect of TF. This finding indicates that TF gives rise to a graded rather than all-or-none response. Indeed, the recipients given TF prepared from 5 or more spleens showed a greater magnitude of response than that observed in antigen-sensitized animals. Ascher et al. (1974) also reported a dose-response effect of TF in the presence of a constant concentration of a specific antigen in vitro. These data suggest that TF exerts its effect on a reservoir of virgin T cells in a recipient to generate a new clone of T cells reactive to a specific antigen within 24 hr and that, in proportion to the dose, it expands the clone, even to such a degree that the clone produced de novo by TF exceeds that in an antigen-sensitized animal.

Gel chromatography and the UV absorption spectrum of TF revealed that it is a substance of ca 1,000 molecular weight, seemingly containing nucleic acids. How can a nucleic acid, too small to code for the protein which is presumably involved in antigen recognition, transmit information for a specific immune response that generates and expands a new clone of specific T cells in such a short time? It is hardly conceivable that a particular population of T cells could proliferate to such an extent that it could elicit a secondary response within 24 hr. We consider it more likely that TF converts a population of T cells preprogrammed to react to a certain antigen into an antigen-responsive state without inducing it to proliferate. Then TF, as Pappenheimer (1957) postulated, might be considered to be a specific T cell receptor itself, which adheres to naive T cells to make it reactive to a specific antigen. An alternative candidate for the identity of TF is, as suggested by Lawrence (1969), a derepressor, which switches on a clone of T cells already destined to respond to a specific antigen.

It was confirmed earlier that, upon contact with a given antigen in vitro, the educated lymphocytes promptly released the monospecific TF (Lawrence and Pappenheimer 1957; Graybill et al. 1973). Feldmann et al. (1973) provided the evidence that the primed carrier-reactive T cells actively shed the antigen-specific T cell factor, which cytaphilically binds to macrophages to help hapten-reactive B cells make an anti-hapten response. In analogy to this, it is likely that the monospecific TF released by the educated lymphocytes converts naive T cells into committed cells in vivo.

We think it teleologically appropriate that TF, whether it be a receptor or a derepressor, is released by the educated lymphocytes which have just reacted to antigens and that it, by converting naive cells, expands the specific clone so as to successfully defend the host against invading pathogens or anything recognized as not-self.

The exact character and the mechanism of action of TF remain to be elucidated.
Acknowledgments

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References


