**MIDDLE-AGED SUBJECTS WITH HABITUAL LOW-SPEED CYCLING EXERCISE HAVE GREATER MONONUCLEAR CELL RESPONSIVENESS AGAINST HUMAN HEPATITIS B VIRUS SURFACE ANTIGEN**

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**SUMMARY**

**Background:** Whether middle-aged people with habitual cycling exercise (HCE) at low intensity in the morning have higher immunity against hepatitis B virus than sedentary controls (SCs) is a health issue in the elderly.

**Methods:** Conditioned media (CM) were prepared by stimulating isolated human peripheral blood mononuclear cells (MNC) with phytohemagglutinin (PHA) or assessment of their inhibitory effects on hepatitis B surface antigen expression in human hepatoma Hep3B cells.

**Results:** With the percent of maximal oxygen uptake at about 45.52% and percent of maximal heart rate at about 68.58% during a cycling exercise program in the present study, we considered HCE as an aerobic and a low to moderate exercise for the elderly. The concentrations of secreted cytokines such as interferon gamma, tumor necrosis factor α and interferon alpha were higher in the MNC-CM from the HCE group than from the SC group. The inhibitory rates of MNC-CM of the HCE group against hepatitis B surface antigen expression were higher than that of the SC group. In the same stimulating concentration of PHA (10 μg/mL), the relative hepatitis B surface antigen expression in MNC-CM of the HCE group was 64.7% versus 81.5% of the SC group. The reduction in inhibitory rates in cytokine neutralization experiments suggests crucial roles of these cytokines for the inhibitory effect of HCE-PHA-MNC-CM against hepatitis B surface antigen expression.

**Conclusion:** The results reveal that the immune response of MNC, which are stimulated by PHA to suppress hepatitis B surface antigen expression, is greater in middle-aged subjects with low-speed HCE than in sedentary subjects. [International Journal of Gerontology 2010; 4(2): 82–88]

**Key Words:** aerobic exercise, hepatitis B surface antigen, hepatitis B virus, immunomodulation

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**Introduction**

In respect of the fossil fuel energy crisis and environmental protection, cycling for leisure or transport is popular in Taiwan. Without sudden impact during exercise, light to moderate aerobic cycling is a healthy alternative exercise for the elderly to avoid injury to the foot or ankle. It has been reported that people who cycle to work 3 hours per week can decrease the risk of mortality by up to 40% when compared with non-participants. In elderly subjects with habitual 15–29 minutes of walking or cycling to work in Finland, a marked decrease in cardiovascular mortality was observed.

Regular, moderate exercise for 60 minutes per week for 12 months in the elderly was found to enhance mucosal immune function, and both the concentration...
and secretory rate of IgA in salivary were increased. It is well known that exercise can modulate immune function, depending on the intensity of exercise. Some studies have mentioned the effect of exercise on cellular immune function in the elderly. For example, habitual elderly endurance exercisers had a greater T-cell function than sedentary controls (SCs) in a cross-sectional survey. Furthermore, elderly women with 16 weeks of endurance training developed greater resting natural killer cell activity compared with SCs.

After moderate exercise intervention, elderly subjects being administered a trivalent influenza vaccine had reduced antibody titer in their blood compared with controls. The greater resistance in mice against infection by herpes simplex virus type 1 was also assumed to be related to moderate exercise.

There are some cytokines associated with immunity against viruses. Interferon gamma (IFN-γ) secreted by human peripheral blood mononuclear cells (PBMNC), was observed to exhibit a marked inhibitory effect on hepatitis B virus (HBV) expression without cytolytic effects in cells. There was some evidence that tumor necrosis factorα (TNF-α) may also be involved in the activation of the cell-mediated antiviral immune response to HBV. The cell-mediated antiviral immune response to HBV in PBMNC in children may be attributed to higher TNF-α production. Exercise can augment the secretion of TNF-α in the plasma of healthy men after moderate exercise.

We proposed that regular aerobic cycling exercise will improve the immune response of phytohemagglutinin (PHA) against HBV expression in human hepatoma Hep3B cells. To evaluate the difference in immune response against HBV expression between middle-aged people performing habitual cycling exercise (HCE) and (SCs), we isolated PBMNC and incubated them with PHA to simulate the immune reaction induced in vivo. Whether any antiviral effect was induced by the secretion of cytokines from PBMNC was also assessed.

Subjects and Methods

Subjects

The human ethics committee of Chinese Culture University authorized the study. Fifteen middle-aged males were recruited and gave their informed consent. Those with HCE lasting 2 hours in the morning at least twice a week for more than 2 years were recruited as the HCE group. Another 14 males with no participation in any other habitual physical activities before the study were recruited as the SC group. All subjects were examined by a clinic physician and were judged to have no clinical history of obvious musculoskeletal illness, pulmonary disease and cardiovascular disease, and were neither HBV carriers nor hepatitis B surface antigen (HBsAg)-positive. Furthermore, they had not had any other surgical treatment during the 4 months prior to blood collection and the experimental period. Before blood collection, the HCE group underwent a HCE program for 2 months. The HCE program was cycling exercise at a speed equivalent to a percent peak oxygen uptake (VO2peak) of 32% from 5:00 to 8:00 in the morning twice a week. Other physical activities were prohibited during the experimental period. All subjects had breakfast, including three slices of white toast, one egg and 240 mL of milk, 30 minutes before the HCE program. Intake of any caffeinated beverages, alcoholic drinks or drugs, smoking, and a vegetarian diet were restricted for 4 weeks before the study, as well as during the experimental period. To ensure the same intensity of exercise in each subject, a heart rate meter (pacer heart rate monitor; Polar, Port Washington, NY, USA) was worn during all the HCE to help maintain the heart rate at a constant rate. Blood samples, before eating and after the subjects had rested quietly for at least 48 hours, were taken between 8:00 and 9:00 in the morning. All subjects of the HCE and SC groups, who had a mean age of 67.0 ± 5.6 years (range, 59–69 years), mean height of 164.2 ± 8.3 cm and mean weight of about 62.1 ± 9.5 kg, were volunteers. The baseline anthropometric data between the HCE and SC groups showed no significant differences (Table 1).

Measurements of cardiopulmonary fitness

Two visits were required by each subject in the preliminary study. The first visit was the measurement of VO2peak and oxygen consumption. The second visit was to determine the cycling speed with a 32% VO2peak during the HCE. The VO2peak and oxygen consumption were measured by a respiratory monitoring system (MetaMax 3B; Cortex Biophysik GmbH, Leipzig, Germany), while the subjects were riding an electronically braked cycle ergometer (Corival 400; Lode, Groningen, The Netherlands) with incremental exercise testing. The test for the VO2peak was completed 1 month before the experimental period. After warming up with preliminary exercise at 75 W for 2 minutes, the subjects had increased loading at 25 W every 2 minutes until volitional fatigue.
Hepatoma cell cultures

We used Hep3B/C16 (Hep3B) cell cultures, which are a human hepatocellular carcinoma cell line with the HBV genome integrated into its chromosome giving stable production of HBsAg, as the cell model for studying HBV replication\textsuperscript{16,17}.

We found that about 30% by volume of mononuclear cell (MNC) conditioned medium (CM) was suitable for Hep3B cells in our preliminary experimental work. The MNC-CM was added into fresh Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal calf serum for incubation of Hep3B cells for 3 days at 37°C in a humidified 5% CO\textsubscript{2} incubator. The Hep3B cells were incubated in Dulbecco’s modified Eagle’s medium, containing 10% fetal calf serum (Invitrogen), streptomycin at 100 mg/L, L-glutamine at 1,000 μmol/L, and penicillin at 10\textsuperscript{5} IU/L, in a humidified 5% CO\textsubscript{2} incubator at 37°C at an initial concentration of 1 × 10\textsuperscript{6} cells/mL. There was no direct effect on the relative HBsAg expression and on the viability of Hep3B cells by a PHA concentration of 10 μg/mL in the MNC-CM. We used the tetrazolium dye MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] colorimetric test to evaluate cell viability\textsuperscript{18}. Hep3B cell medium, which was incubated with 30% phosphate-buffered saline containing PHA at 10 μg/mL, replaced the MNC-CM and represented the untreated control group. Cell viability was calculated by dividing the MTT value of Hep3B treated with MNC-CM from the HCE group by the MTT value of Hep3B treated with MNC-CM from the control group. Cell viability was calculated by dividing the MTT value of MNC-CM untreated control group. Cell viability was calculated by dividing the MTT value of Hep3B treated with MNC-CM from the HCE group by the MTT value of Hep3B treated with MNC-CM from the control group.

Preparation of MNC-CM

PHA, a natural mitogen of T lymphocytes, has been used as an immunostimulant in many studies to simulate the immune response and effect. To simulate the immune reaction for evaluation of drug effects\textsuperscript{19,20}, PBMC from human blood, which was stimulated with PHA at 10 μg/mL, was used as a model system. Articles on exercise-mediated immunomodulation using the above model have been published\textsuperscript{21,22}. In this model system, various cytokines released from PBMC by PHA stimulation have been studied. MNCs were isolated from the peripheral blood of each subject by Ficoll-Hypaque solution (1.077 g/mL; Pharmacia Biotech, Uppsala, Sweden) with density centrifugation (400 g for 30 minutes)\textsuperscript{23}. MNCs were washed by phosphate-buffered saline three times and suspended in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated autoserum, then incubated in autoserum-coated culture plates. Cells were cultured in 10% heat-inactivated fetal calf serum (Gibco; Invitrogen) at an initial concentration of 1 × 10\textsuperscript{6} cells/mL, with L-glutamine (Invitrogen) at 1,000 μmol/L, streptomycin (Invitrogen) at 100 mg/L, penicillin (Invitrogen) at 50 mg/L, and RPMI 1640 medium containing PHA (Sigma-Aldrich) at 10 μg/mL, at 37°C in a fully humidified incubator with 5% CO\textsubscript{2}. The collected aliquot, which had all the MNCs removed by filtering through a 0.45 μm membrane after cultivation for 24 hours was named the CM, and stored at −80°C until use\textsuperscript{23}. PHA-MNC-CM was prepared with PHA at 10 μg/mL for 24 hours to observe the relative HBsAg expression in Hep3B cells. All of the MNC-CM were collected to measure the secretion of cytokines. The MNC-CM included cytokines related to antiviral and antitumor immunity such as IFN-γ, TNF-α, and interferon alpha (IFN-α). PHA-MNC-CM from the SC group was termed as SC-PHA-MNC-CM and from the HCE group as HCE-PHA-MNC-CM.

Assay for cytokines

In the cytokine-neutralizing experimental procedure, HCE-PHA-MNC-CM were preincubated with various cytokine-neutralizing antibodies, including anti-IFN-γ (30.0 μg/mL, which was tenfold more than the concentration at near 100% neutralization), anti-TNF-α (2.4 μg/mL), anti-IFN-α (1.0 μg/mL) and anti-interleukin (IL) 1β antibodies (5.1 μg/mL), alone or in combination at 37°C for 90 minutes. After 48 hours’ incubation with added antibodies of the cytokines, all viable cells were measured. Three separate tests were each performed in duplicate. The commercial enzyme-linked immunosorbent assay (ELISA) kits, which include IFN-γ, IFN-α, TNF-α and IL-1β (R&D Systems, Minneapolis, MN, USA), were for measurement at a wavelength of 450 nm to determine the amount of secreted cytokines in PHA-MNC-CM.
by following the method described by Wang et al. The correlation coefficients \( r^2 \) for the standard curves of the above four cytokines were between 0.998 and 0.999.

**Assay for relative HBsAg expression**

As the presence of HBsAg in serum of HBV virus carriers indicates a current HBV infective status and a risk of developing compensated cirrhosis and hepatocellular carcinoma, HBsAg is a common indicator of HBV activity.

The Hep3B cell line was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum for 24 hours to proliferate to over \( 1 \times 10^6 \) cells/mL, and were subsequently transferred into serum-free Dulbecco's modified Eagle's medium with or without 30% (vol/vol) MNC-CM at \( 1 \times 10^6 \) cells/mL for 48 hours incubation. Commercial ELISA kits (General Biological, Taipei, Taiwan) were used to determine the amount of secreted HBsAg in the cultured CM. By preliminary test, we determined that PHA did not interfere with the HBsAg assay by the ELISA kits. The measured optical density values of the ELISA kits during measurement were normalized to cell numbers. The relative HBsAg expression was calculated as \( \frac{\text{HBsAg/MTT}}{} \) from PHA-MNC-CM of the HCE group divided by \( \frac{\text{HBsAg/MTT}}{} \) from the untreated control group culture media. The secreted HBsAg amount per viable Hep3B cell (i.e., HBsAg/MTT) from the untreated control group culture media was treated as 100% expression.

**Statistical analysis**

Results are presented as mean±standard error of the mean. Differences between the treatment groups, which consisted of matched samples, were assessed by the Student t test. A confidence level of 5% \( (p<0.05) \) was considered significant.

**Results**

**Anthropometric measurement and \( V\Omega_{2\text{peak}} \) of subjects**

The HCE group had a mean body weight of 59.4±9.2 kg (range, 46–74 kg), mean age of 67.6±5.4 years (range, 49–78 years), and mean height of 162.5±10.1 cm (range, 153–176 cm). The SC group had a mean age of 66.3±5.8 years (range, 51–76 years). There were no differences in the anthropometric measurements between the SC group and the HCE group \( (p>0.05) \) as shown in Table 1. The \( VO_{2\text{peak}} \) observed in the HCE group, 25.6±7.1 mL/kg·min⁻¹, was similar to that in the SC group, 24.2±6.7 mL/kg·min⁻¹ \( (p>0.05) \). From Table 2, the respiratory exchange ratios before and after HCE showed no noticeable change. The lactate concentration in serum (1.68–1.98 mmol/L) showed no marked change. The changes in intensity of HCE were minor, from the data of percent of \( VO_{2\text{peak}} \) (21.26%±12.73% to 45.52%±13.28%) and percent of maximal heart rate (49.87%±6.82% to 68.58%±6.71%). The metabolic equivalents were similar before and after HCE.

**Comparison of the reduction of relative HBsAg expression in Hep3B cells stimulated by PHA-MNC-CM between the SC group and HCE group**

After stimulation by PHA, a much greater response was observed in HCE-PHA-MNC-CM than in SC-PHA-MNC-CM. There was markedly lower HBsAg expression of 64.7% in Hep3B cells incubated with HCE-PHA-MNC-CM in comparison to 81.5% with SC-PHA-MNC-CM (Table 3). No apparent inhibition of relative HBsAg expression in Hep3B cells was observed in the SC group. It should be noted that no obvious Hep3B cell death was present in either group at a PHA concentration of 10 μg/mL.

**Effects of cytokines on HBsAg expression in Hep3B cells**

PHA-stimulated secretion of all the cytokines, IL-1β, TNF-α, IFN-γ and IFN-α, in HCE-PHA-MNC-CM markedly increased to 876±211 pg/mL, 1,293±289 pg/mL, 712±125 pg/mL and 1,057±229 pg/mL, respectively, compared with those in SC-PHA-MNC-CM to 312±134 pg/mL, 621±251 pg/mL, 387±98 pg/mL and 363±158 pg/mL, respectively (Table 4).

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**Table 2. Blood lactate concentration and energy expenditure before and after habitual cycling exercise (HCE)**

<table>
<thead>
<tr>
<th></th>
<th>Before HCE</th>
<th>During HCE</th>
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<tbody>
<tr>
<td>Blood lactate (mmol/L)</td>
<td>1.98±0.39</td>
<td>1.68±0.46</td>
</tr>
<tr>
<td>%HRmax (%)</td>
<td>49.87±6.82</td>
<td>68.58±6.71</td>
</tr>
<tr>
<td>%VO₂peak (%)</td>
<td>21.26±12.73</td>
<td>45.52±13.28</td>
</tr>
<tr>
<td>MET</td>
<td>1.3±1.1</td>
<td>4.3±1.3</td>
</tr>
<tr>
<td>RER</td>
<td>0.76±0.08</td>
<td>0.75±0.08</td>
</tr>
</tbody>
</table>

*Data are expressed as mean±standard error of the mean. %HRmax=percent of maximal heart rate achieved during incremental exhaustive exercise; %VO₂peak=percent of peak oxygen uptake; MET=metabolic equivalent; RER=respiratory exchange ratio.
Table 3. Effects of phytohemagglutinin (PHA)-containing mononuclear cell (MNC) conditioned medium (CM) and cytokine antibody neutralization on the relative hepatitis B surface antigen (HBsAg) expression*  

<table>
<thead>
<tr>
<th></th>
<th>Relative HBsAg expression (%)</th>
<th>MTT cell viability assay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>SC-PHA-MNC-CM</td>
<td>81.5 ± 13.7</td>
<td>99.1 ± 12.4</td>
</tr>
<tr>
<td>HCE-PHA-MNC-CM†</td>
<td>64.7 ± 12.8</td>
<td>97.5 ± 13.3</td>
</tr>
<tr>
<td>+ anti-IL-1β antibodies‡</td>
<td>67.3 ± 12.5</td>
<td>97.2 ± 11.8</td>
</tr>
<tr>
<td>+ anti-IFN-α antibodies§</td>
<td>82.5 ± 11.7</td>
<td>99.1 ± 11.4</td>
</tr>
<tr>
<td>+ anti-TNF-α antibodies¶</td>
<td>78.7 ± 13.8</td>
<td>97.3 ± 12.8</td>
</tr>
<tr>
<td>+ anti-TNF-α + anti-IFN-α</td>
<td>79.3 ± 13.2</td>
<td>99.1 ± 13.5</td>
</tr>
<tr>
<td>+ anti-IFN-γ antibodies§</td>
<td>87.1 ± 11.4</td>
<td>98.2 ± 11.3</td>
</tr>
</tbody>
</table>

*Triplicated data from separate experiments are expressed as mean ± standard error of the mean; ‡ aliquots were preincubated, with or without cytokine-neutralizing antibodies, at 37°C for 90 minutes before addition to Hep3B cell culture; † 5.1 μg/mL; § 30.0 μg/mL; ¶ 2.4 μg/mL; γ MTT = (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide); Sc = sedentary control; HCE = habitual cycling exercise; IL-1β = interleukin 1β; IFN-α = interferon alpha; TNF-α = tumor necrosis factor α.

relative HBsAg expression in Hep3B cells decreased to a lower level of 67.3% ± 12.5% with pretreatment of anti-IL-1β antibodies in HCE-PHA-MNC-CM; there was a small, statistically significant increase in relative HBsAg expression when compared with HCE-PHA-MNC-CM alone (64.7% ± 12.8%). The relative HBsAg expression in Hep3B cells increased to 78.7% ± 13.8% in the presence of anti-IFN-α antibodies, to 82.5% ± 11.7% in the presence of anti-IFN-γ antibodies, and to 79.3% ± 13.2% in the presence of anti-TNF-α antibodies (Table 3). With a combination of all three cytokine antibodies, the greatest effect was seen with the relative HBsAg expression in Hep3B cells being 87.1% ± 11.4%.

Discussion

The analysis of significant inhibition of HBsAg expression in an ex vivo experimental model has shown a greater immunomodulatory response of PBMC isolated from middle-aged people with HCE than the SCs.

From the basic anthropometric data, the HCE group and SC group were similar. However, there was a statistically greater VO2peak in the HCE group than in the SC group. The percent of maximal heart rate during HCE at about 68.58% can be defined as moderate exercise. The other strong evidence to define HCE as moderate intensity exercise was the fact that %V·O2peak after HCE was about 45.52% and metabolic equivalent was 4.3. From the lack of change in blood lactate with exercise duration, we can refer to the HCE program as aerobic exercise of low to moderate intensity.

The secreted cytokines, mainly TNF-α, IFN-α, and IFN-γ, dominated the inhibitory effects of HCE-PHAPHAMNC-CM. To enhance the response of antiviral immunity, moderate-intensity cycling exercise might be a possibility. Moderate exercise training could diminish the activity of influenza virus as shown in another in vivo study28. After removal of MNCs, the soluble mediators produced by MNCs in CM rather than the MNC cells themselves may be the major factors causing inhibition of HBsAg expression in Hep3B cells.

The much greater amounts of IFN-α, TNF-α and IFN-γ secreted in HCE-PHA-MNC-CM than in SC-PHAPHAMNC-CM indicate a greater response of MNC in the subjects taking moderate cycling exercise. The neutralization by anti-IFN-γ, anti-TNF-α and/or anti-IFN-α antibodies in HCE-PHAPHAMNC-CM caused the partial loss of the inhibitory activity against relative HBsAg expression.
The results of antibody neutralization showed that soluble factors in CM, including IFN-γ, IFN-α and TNF-α, may contribute to the greater inhibitory activity against HBsAg expression in HCE-MNC-CM. It should be noted that both the MNC and Hep3B cells exhibited no cytotoxicity (data not shown). Therefore, the greater inhibitory activity exerted by HCE-MNC-CM in our study came from the cytokines rather than from the cytotoxicity of Hep3B cells.

It was reported that active, habitual older runners with a moderate exercise habit exhibited greater blood IFN-γ concentration after stimulation by PHA than was found in sedentary older people. The effects of IFN-γ on HBsAg inhibition of patients has also been well documented in some clinical studies. The obviously lower relative HBsAg expression after anti-IFN-γ antibody neutralization and higher amount of secreted IFN-γ in HCE-PHA-MNC-CM both provide evidence to suggest an important inhibitory role played by IFN-γ. Similar evidence has been presented in other studies, in which there was a negative correlation between cell cytoplasmic HBV DNA and the secreted amount of IFN-γ in the medium of PBMNC.

TNF-α is well known to be an initial activator of antiviral effects in the immune system. For example, the administration of TNF-α into the medium led to suppression of HBV DNA replication in hepatocytes without cytotoxic effects. An apparent reduction in HBV replication was observed after addition of TNF-α to the culture of Hep3B cells. Both findings regarding the greater secretion of TNF-α and the lower relative HBsAg expression with anti-TNF-α antibody neutralization in HCE-PHA-MNC-CM provide evidence to suggest that TNF-α plays a substantial role.

IFN-α has been used in clinical therapy for patients to inhibit virus replication. IFN-α administered to hepatitis B e antigen-positive patients diminished hepatitis B e antigen in the serum from 40% to 25%. We can presume that IFN-α exerts important effects on the inhibition of HBV, because there was a much greater amount of secreted IFN-α and lower relative HBsAg expression after the neutralization of anti-IFN-α antibody in HCE-PHA-MNC-CM.

In addition, evidence on the antiviral activity of immunomodulatory cells activated by increased secretion of IFN-α in a similar experimental model has been presented. For instance, the neutralization by specific antibody of IFN-α in MNCs reduced the suppressant effects against herpes simplex viruses.

Although greater secretion of cytokines was closely related to the reduction in HBsAg expression, the Hep3B cells, which are the HBV-harboring host, were not killed by HCE-PHA-MNC-CM. It can, therefore, be postulated that the inhibitory activity of HCE-MNC-CM against HBsAg expression may not be attributed to the cytotoxicity of Hep3B cells. Both IFN-γ and TNF-α secreted from HBV-specific cytotoxic T lymphocytes can reduce HBV gene expression and replication without the occurrence of cytopathy.

After incubation with a combination of anti-TNF-α, anti-IFN-α and anti-IFN-γ antibodies, the inhibitory activity against HBsAg expression in HCE-PHA-MNC-CM was most attenuated. Thus, IFN-γ, IFN-α and TNF-α, induced by HCE in our study played the crucial immunomodulatory antiviral role.

In conclusion, a greater immunomodulatory response against HBsAg expression of a HBV cell line existed in the HCE group as shown by our ex vivo antiviral immunity model. The much greater secretion of cytokines, mainly IFN-γ, TNF-α and IFN-α from human peripheral blood MNC, contributed to the greater immunomodulatory response against HBsAg expression of a HBV cell line by HCE in middle-aged people.

Acknowledgments

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References


