Decrease in Generation of Reactive Oxygen Species by Neutrophils From Patients With Infectious Mononucleosis: Role ofSuppressor T Lymphocytes

By Yukie Niwa, Tsuyoshi Sakane, Yoshiki Miyachi, Tadashi Kanoh, and Kyoichi Somiya

We assessed the generation of reactive oxygen species (ROS: O_2^-, H_2O_2, OH\cdot, chemiluminescence) by neutrophils and monocytes from six patients with infectious mononucleosis, ten patients with other viral diseases, and ten normal controls. Neutrophils from infectious mononucleosis patients showed markedly decreased generation of all reactive oxygen species, compared with the two control groups; this abnormality persisted for four to eight weeks after disease onset. Monocytes from these patients generated normal levels of ROS. Normal neutrophils incubated with T lymphocytes from infectious mononucleosis patients generated significantly less of each ROS than did those incubated with T cells from either control group. T cell-mediated suppression of ROS generation required both OKT4+ cells from infectious mononucleosis patients and OKT8+ cells from either patients or normals. We conclude that the generation of reactive oxygen species in neutrophils is suppressed in patients with infectious mononucleosis, at least in part, by interacting subsets of T lymphocytes.

The controls were ten sex- and age-matched healthy volunteers and ten patients with influenza, varicella (chicken pox), or herpes zoster (cutaneous herpes zoster). The diagnosis of influenza was made by the hemagglutination inhibition test, and that of varicella or herpes zoster by the complement fixation test. Informed consent forms were obtained from all of the subjects tested before the study. No subject had taken any medication 48 hours before the test. All tests were performed within three days after the onset of symptoms, and studies of IM patients were repeated at biweekly intervals until the values returned to normal.

Assays of ROS Generation

PMNs were isolated from peripheral blood by Ficoll-Hypaque gradient centrifugation as previously described. Measurements of O_2-, H_2O_2, and OH\cdot were carried out in Krebs-Ringer phosphate buffer (KRP), as previously described. Briefly, formation of O_2 was determined by ferricytochrome c (type III, Sigma Chemical Co., St Louis) reduction by O_2 produced from 4 × 10^6 PMNs stimulated with 1 mg/mL opsonized zymosan (Sigma); absorbance was measured at 550 nm.

Generation of H_2O_2 was determined by using 2.5 × 10^6 PMNs stimulated with 1 mg/mL opsonized zymosan, 0.1 mL of 50 mmol/L scopoletin (Sigma) in KRP buffer, and 0.1 mL of 1 mg/mL horseradish peroxidase (type II, Sigma): the rate of decrease in fluorescence intensity of the scopoletin within 30 minutes was measured in a fluorescence spectrophotometer (Hitachi Co, Ltd, Tokyo).

Hydroxyl radical (OH\cdot) was measured by the amount of ethylene gas formed from the reaction of α-keto-methyl butylic acid (Sigma) with the PMN-generated OH\cdot: 2 × 10^6 PMNs stimulated with 1 mg/mL opsonized zymosan and 1 mmol/L α-keto-methyl butylic acid were used, and the total amount of ethylene gas formed at 10, 20, and 30 minutes was determined on a gas-chromatograph (Hitachi).

Chemiluminescence was measured in a scintillation spectrometer (Packard, Downers Grove, Ill), as previously described. Five million PMNs in 3 mL Hanks solution containing 1 mg/mL opsonized zymosan, but no luminol, were incubated in the dark. Chemiluminescence was monitored on the spectrometer, which was operated in the out-of-coincidence summation mode.

The specificity of each assay was confirmed by the depletion of each ROS by its specific scavenger (400 U/mL superoxide dismutase [SOD] for O_2-, 600 U/mL catalase for H_2O_2, 10 mmol/L benzotriazole for OH\cdot, and 2 mmol/L/L xanthine for chemiluminescence). SOD and catalase, heated at 130 °C for 30 minutes, had no effect on the generation of O_2- and H_2O_2, respec-

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tively. In each ROS assay system, PMNs were simultaneously tested with stimulation of 1 μg/mL phorbol myristate acetate (PMA) instead of 1 mg/mL opsonized zymosan, or without any stimulation.

**T Lymphocyte and Monocyte Preparations**

Peripheral blood samples from each subject were divided into two portions; PMNs were harvested from one portion and mononuclear cells from the other.67 Thereafter, adherent (monocytes) and nonadherent cells (lymphocytes) were further separated from the mononuclear cell fractions by incubation in Petri dishes, by the method of Reiss and Roos.6 Adherent cell preparations were identified as 95% monocytes by Giemsa staining and by reaction with OKM1 monoclonal antibody (Ortho Pharmaceutical Corp, Raritan, NJ), which binds to cells of the monocyte and myeloid series. Monocytes from the patients and controls were also assessed for ROS generation, as described for PMNs. The T lymphocyte fraction was isolated by the sheep erythrocyte rosetting technique68 and contained less than 1% of the purified subpopulations at 37 °C for 24 hours did not affect the expression of the OKT4 or OKT8 cell surface antigens. In some experiments, the purified T cell suspensions were enriched for OKT4+ or OKT8+ cells, respectively, by repetitive treatment of the cells with the monoclonal antibodies OKT8 or OKT4 (Ortho Pharmaceutical) and rabbit complement.69 Purity of each negatively selected T cell subpopulation exceeded 90%, as assessed by indirect immunofluorescence using fluoresceinated rabbit anti-mouse IgG (Miles Laboratories, Inc, Palo Alto, Calif) and a fluorescence microscope (Nihon Kagaku, Inc, Tokyo). Culture of the purified subpopulations at 37 °C for 24 hours did not affect the expression of the OKT4 or OKT8 cell surface antigens.

**Incubation of PMNs With T Lymphocytes**

To examine the effect of T cells on PMNs, purified T lymphocytes or T lymphocyte subsets from the patients or controls, supplemented with 5% mitomycin (MMC) treated monocytes, were coincubated with PMNs from healthy individuals at 37 °C for 17 hours. Previous studies2,3 and preliminary experiments in the present study showed that 17 hours is the optimal duration of PMN-lymphocyte coculture for inducing changes in ROS generation without inducing a decrease in the viability and phagocytic functions of PMNs. Before and after coincubation with T lymphocytes, the viability of PMNs was assessed, and related data were discarded when PMNs showed less than 98% viability by trypan blue exclusion and fewer than 600 dpm/mg protein 14C-inulin uptake as a measurement of phagocytic activity.2,3,13 The effect of ROS generated in T cell-PMN cocultures by a small amount of added or contaminating monocytes was negligible, because monocytes generate far less ROS than PMNs do.5 The coculture ratio of PMNs to T lymphocytes was 2:1 when suspended in RPMI 1640 medium containing 20% heat-inactivated pooled human AB serum. Thereafter, PMNs isolated from T lymphocytes by the Ficoll-Hypaque gradient method or PMNs in the presence of T lymphocytes were examined for ROS generation as already described. Control incubations (17 hours, 37 °C) of T lymphocytes, the viability of PMNs was assessed, and related data were discarded when PMNs showed less than 98% viability by trypan blue exclusion and fewer than 600 dpm/mg protein 14C-inulin uptake as a measurement of phagocytic activity.2,3,13 The effect of ROS generated in T cell-PMN cocultures by a small amount of added or contaminating monocytes was negligible, because monocytes generate far less ROS than PMNs do.5

**Statistical Analysis**

Triplicate assays were performed simultaneously for each experiment, and the results were expressed as mean ± SE. The statistical significance was ascertained by the Student's t test.

**RESULTS**

**ROS Generation by Phagocytes From the Patients**

Opsonized zymosan-stimulated PMNs from IM patients showed markedly decreased generation of ROS, compared with both diseased and healthy control groups (O2; H2O2; .01 < P < .05; chemiluminescence: P < .01; OH·: P < .001) (Table 1). On the other hand, no decrease in ROS generation was observed in monocytes from IM patients. Although monocytes generated lower ROS levels than PMNs in both control groups, IM patients showed almost equal levels of ROS generation by their PMNs and monocytes. ROS generation by PMNs from patients with other viral infections was only slightly decreased, which was not significant when compared to healthy controls (P > .05).

When ROS generation by unstimulated PMNs or by PMA-stimulated PMNs was assayed, similar findings to those made with opsonized zymosan-stimulated PMNs were obtained, although the changes of H2O2 by PMNs were more marked. There was a marked difference between ROS production by unstimulated PMNs from IM patients and both healthy and diseased controls (O2, H2O2; .01 < P < .05; chemiluminescence: P < .01; OH·: P < .001). PMA-stimulated neutrophils from IM patients also generated markedly decreased ROS levels as compared with both healthy and diseased controls (O2, H2O2; .01 < P < .05; OH·, chemiluminescence: P < .01; H2O2: P < .001) (Table 1).

**Coincubation of Normal PMNs With Patients' Lymphocytes**

PMNs from healthy individuals following incubation with T lymphocytes from IM patients generated markedly decreased ROS levels compared with PMNs incubated with T lymphocytes from allogeneic healthy individuals (O2; chemiluminescence: .01 < P < .05; H2O2; P < .01; OH·: P < .001). However, incubation with T lymphocytes from patients with other viral infections did not induce a decrease in ROS generation by PMNs from healthy individuals (P > .05) compared to incubation with normal T lymphocytes (Table 2). PMNs separated from T lymphocytes following coincubation generated approximately the same levels of ROS as were generated by PMNs in the presence of T lymphocytes (Table 3). Lymphocytes alone from each subject failed to generate measurable ROS (data not shown).
Table 1. Generation of Reactive Oxygen Species by PMNs and Monocytes From IM Patients and Control Groups With or Without Stimulation of Opsonized Zymosan or PMA

<table>
<thead>
<tr>
<th></th>
<th>IM Patients</th>
<th>Patients With Varicella, H-Z, and Influenza Healthy Controls</th>
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<tbody>
<tr>
<td></td>
<td>PMNs (N = 6)</td>
<td>Monocytes (N = 6)</td>
</tr>
<tr>
<td>O₂ (pmol x 10³/min/4 x 10⁸ PMNs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3.24 ± 0.28</td>
<td>3.78 ± 0.27</td>
</tr>
<tr>
<td>B</td>
<td>3.01 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.16 ± 0.004</td>
<td></td>
</tr>
<tr>
<td>H₂O₂ (pmol x 10³/min/2.5 x 10⁸ PMNs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3.03 ± 0.19</td>
<td>3.62 ± 0.23</td>
</tr>
<tr>
<td>B</td>
<td>4.75 ± 0.11†</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.87 ± 0.06†</td>
<td></td>
</tr>
<tr>
<td>OH⁻ (pmol x 10²/2 x 10⁸ PMNs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3.01 ± 0.24†</td>
<td>2.73 ± 0.18</td>
</tr>
<tr>
<td>B</td>
<td>2.76 ± 0.19‡</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.035 ± 0.003‡</td>
<td></td>
</tr>
<tr>
<td>Chemiluminescence (cpm x 10⁵/5 x 10⁸ PMNs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10.4 ± 0.34‡</td>
<td>9.40 ± 0.61</td>
</tr>
<tr>
<td>B</td>
<td>74.320 ± 6.824</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1.45 ± 0.09†</td>
<td></td>
</tr>
</tbody>
</table>

(A) The levels of ROS generated with stimulation of 1 mg/mL opsonized zymosan. (B) The levels of ROS generated with stimulation of 1 µg/mL PMA. (C) The levels of ROS generated without any stimulation.

‡P < .001 v healthy controls.
†P < .01 v healthy controls.
§01 < P < .05 v healthy controls.

The number of monocytes used for ROS assay was the same as that of PMNs.

Table 2. Opsonized Zymosan-Stimulated Generation of Reactive Oxygen Species by PMNs From Healthy Individuals After 17-Hour Incubation With T Lymphocytes From IM Patients and Control Groups

<table>
<thead>
<tr>
<th>Incubation With T Lymphocytes From</th>
<th>Patients (N = 6)</th>
<th>Patients With Varicella, H-Z, and Influenza Healthy Controls (N = 7)</th>
<th>Allogeneic Healthy Individuals (N = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂ (pmol x 10³/min/4 x 10⁸ PMNs)</td>
<td>2.82 ± 0.23§</td>
<td>4.24 ± 0.41</td>
<td>4.85 ± 0.72</td>
</tr>
<tr>
<td>H₂O₂ (pmol x 10³/min/2.5 x 10⁸ PMNs)</td>
<td>2.10 ± 0.22†</td>
<td>4.09 ± 0.33</td>
<td>4.34 ± 0.52</td>
</tr>
<tr>
<td>OH⁻ (pmol x 10²/2 x 10⁸ PMNs)</td>
<td>3.13 ± 0.40‡</td>
<td>7.01 ± 0.58</td>
<td>7.21 ± 0.63</td>
</tr>
<tr>
<td>Chemiluminescence (cpm x 10⁵/5 x 10⁸ PMNs)</td>
<td>10.20 ± 0.86§</td>
<td>14.00 ± 1.23</td>
<td>16.70 ± 1.29</td>
</tr>
</tbody>
</table>

It was ascertained that ROS levels generated by lymphocytes from any subject alone were negligible.

‡P < .001 v control (ROS levels generated by normal PMNs following coincubation with T lymphocytes from allogeneic healthy individuals).
†P < .01.
§01 < P < .05.

Table 3. Opsonized Zymosan-Stimulated Generation of Reactive Oxygen Species by PMNs From Healthy Individuals After 17-Hour Incubation With Autologous or Allogeneic T Lymphocytes

<table>
<thead>
<tr>
<th>Incubation With T Lymphocytes From</th>
<th>Allogeneic Healthy Individuals (N = 7)</th>
<th>Autologous Individuals (N = 5)</th>
<th>Unseparated Leukocytes* (N = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂ (pmol x 10³/min/4 x 10⁸ PMNs)</td>
<td>4.85 ± 0.72</td>
<td>4.96 ± 0.51</td>
<td>4.62 ± 0.53</td>
</tr>
<tr>
<td>H₂O₂ (pmol x 10³/min/2.5 x 10⁸ PMNs)</td>
<td>4.34 ± 0.52</td>
<td>3.90 ± 0.69</td>
<td>3.98 ± 0.61</td>
</tr>
<tr>
<td>OH⁻ (pmol x 10²/2 x 10⁸ PMNs)</td>
<td>7.21 ± 0.63</td>
<td>7.43 ± 0.88</td>
<td>6.86 ± 0.74</td>
</tr>
<tr>
<td>Chemiluminescence (cpm x 10⁵/5 x 10⁸ PMNs)</td>
<td>16.70 ± 1.29</td>
<td>15.90 ± 2.00</td>
<td>14.30 ± 2.33</td>
</tr>
</tbody>
</table>

There was no statistically significant difference between any two groups.

*ROS generation was assessed for PMNs that were unseparated from mononuclear fractions. In this assay, the proportion of PMNs to T lymphocytes was almost 3:1.
PMN-T lymphocyte coculture.

Characteristics of Suppressor T Cells

Non-T cell populations from IM patients did not cause a decrease in ROS generation by normal PMNs (data not shown). Furthermore, irradiation of T cells from IM patients with doses exceeding 1,500 rad abrogated their capacity to decrease ROS generation by PMNs (Table 4). This suggests that a decrease in ROS generation induced by coincubation with T lymphocytes may be due to inhibition by suppressor T cell activity in IM patients.

Determination of T Cell Subpopulations in Relation to Suppressing Activity on ROS Generation by PMNs

As shown in Table 5, coincubation of normal PMNs with OKT4+ cells from IM patients in the presence of either IM patient-derived or normal OKT8+ cells suppressed ROS generation by normal PMNs. In contrast, normal OKT4+ cells did not suppress ROS generation by normal PMNs even in the presence of OKT8+ cells derived from IM patients. Thus, for T cell-mediated suppression of ROS generation by PMNs, both OKT4+ cells from IM patients and OKT8+ cells appear to be necessary.

PMNs from healthy individuals incubated with T lymphocytes from allogeneic healthy individuals generated almost the same levels of ROS as did PMNs after coculture with autologous T lymphocytes (Table 3). This observation rules out the possibility that the decrease of ROS generation influenced by T lymphocytes was due to HLA differences between the PMN and lymphocyte donors.

Decreased ROS generation by PMNs from IM patients returned to normal values within eight weeks of disease onset, as shown in Fig 1.

DISCUSSION

In studies of patients with infectious mononucleosis due to EBV, T cell-mediated inhibition of B cell immunoglobulin production and lymphoblastoid transformation has been well documented.14 However, although neutropenia is frequently observed in patients with IM, no investigation of neutrophil function in IM has hitherto been reported. It therefore seems notewor-
that, in the present study, the production of reactive oxygen metabolites by neutrophils from patients with infectious mononucleosis was found to be markedly diminished. In IM patients, ROS generation not only by opsonized zymosan-stimulated PMNs, but also by unstimulated PMNs or by soluble stimulant, PMA-stimulated PMNs, showed similarly decreased levels when compared with both diseased and healthy control groups. This indicates that the defect observed in ROS generation by neutrophils from IM patients is not due to modulation of the C3b receptor alone, but to intrinsic alterations of oxidative metabolism. Of further interest is the finding that T cells from IM patients suppressed the generation of ROS by neutrophils. This suppression was not only by opsonized zymosan-stimulated PMNs, but also by unstimulated PMNs or by soluble stimulant, PMA-stimulated PMNs, showed similarly decreased levels when compared with both diseased and healthy control groups. This indicates that the defect observed in ROS generation by neutrophils from IM patients is not due to modulation of the C3b receptor alone, but to intrinsic alterations of oxidative metabolism. Of further interest is the finding that T cells from IM patients suppressed the generation of ROS by neutrophils.

At least two T cell subsets were required for suppression of ROS generation by PMN, but it was only necessary that the OKT4 subset be derived from IM patients. These data suggest that EBV infection elicits OKT4 T cells that either induce OKT8 cells to suppress ROS generation or, in the presence of OKT8 cells, themselves serve as suppressors of ROS generation by neutrophils.

In EBV infection, in which the B cell compartment is the target of infection, T cell-mediated suppression of B cell proliferation and activation apparently serves as an important element of host defense in limiting the infection. Activation of neutrophils to generate ROS does not appear to be an important mechanism of defense against viral infections (Solberg et al. and unpublished data from our laboratory). It is conceivable that the same mechanism by which T cells limit the extent of EBV infection of B cells coincidentally, but innocuously, suppresses PMN function. However, the ability of T cells from convalescent IM patients to suppress EBV-infected B cell outgrowth persists for years; in contrast, the recovery of ROS generation by PMNs occurred within eight weeks of onset of IM in our patients, a time course similar to that seen in the recovery from T cell-mediated suppression of pokeweed-stimulated immunoglobulin synthesis in IM patients and from a variety of immunologic abnormalities in patients with rubella and other viral diseases. It is therefore more likely that T cell-mediated suppression of ROS generation by PMNs in IM involves mechanisms or T cell subpopulations different from those specifically suppressing EBV-induced B cell outgrowth.

It remains to be determined whether ROS generation by PMNs is also suppressed in other clinical states involving EBV infection, such as Burkitt’s lymphoma or nasopharyngeal carcinoma. It is conceivable that chronic suppression of ROS generation may play a pathogenic role in these conditions. Another problem to be elucidated is whether or not decreased ROS generation by PMNs has biologic significance, perhaps playing a pathogenic role in these clinical conditions.

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REDUCED ACTIVE OXYGENS IN EBV INFECTION


Decrease in generation of reactive oxygen species by neutrophils from patients with infectious mononucleosis: role of suppressor T lymphocytes

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