Digestive Diseases and Sciences, Vol. 33, No. 8 (August 1988), pp. 951–955

Active Oxygen Species Generated by Monocytes and Polymorphonuclear Cells in Crohn's Disease

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Chemiluminescence (CL) analysis of monocytes and polymorphonuclear cells (PMNs) was performed on 13 patients with Crohn's disease (CD) and 10 healthy volunteers. The percentages of monocyte populations in mononuclear cells obtained from the patients with CD were greater than those from the healthy volunteers, but the numbers of PMNs were not different between the two groups. The peak level of phorbol myristate acetate (PMA) -induced CL activity generated by diluted whole blood from the patients with CD was more significantly elevated than that from the healthy volunteers, whereas the peak levels of opsonized zymosan-induced CL activity did not differ between the two groups. In monocytes, the peak levels of both PMA- and opsonized zymosan-induced CL activity were significantly higher in the patients with CD than in the healthy volunteers. CL in PMNs, however, showed no significant difference between CD and controls. It is suggested that monocytes of CD have a large capacity to generate active oxygen species. The present study suggests that excessive active oxygen species released by monocytes and perhaps macrophages may play an important role in formation of the intestinal lesions in CD.

KEY WORDS: Crohn's disease; monocyte; chemoluminescence; zymosan; active oxygen species.

Epitheloid cell granulomas are a predominant and distinguishing feature of the histological findings in Crohn's disease (CD). In chronic granulomatous disease (CGD), similar histological lesions to CD were found in the gastrointestinal tract (1), and leukocyte dysfunction has been established as a cause of this pathological condition (2, 3).

In recent years, leukocyte function in CD has also been investigated. In studying mobilization, a defective neutrophil function in CD was found on observation by a skin-window technique *in vivo*, whereas chemotaxis *in vitro* was normal (4–6). Utilizing other methods of determining neutrophil function, impaired, normal, or enhanced function have been reported (5–10). The previous studies were performed mainly in neutrophil function.

Furthermore, it has been demonstrated that leukocyte dysfunction in CGD was a result of defective production of active oxygen species (11)

Manuscript received August 14, 1986; revised manuscript received October 1, 1987; accepted October 26, 1987.

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This work was supported by the Grant of Tokuteishitsukan from the Japanese Ministry of Welfare and Health.

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0163-2116/88/0800-0951$06.00/0

and that macrophages in the tissue were derived from circulating monocytes (12). This study was, therefore, performed to clarify the function of monocytes and polymorphonuclear cells from patients with CD with respect to the generation of active oxygen species.

MATERIALS AND METHODS

Patients and Controls. Thirteen patients with CD, nine males and four females, with a mean age of 28.8 years, were studied. Diagnosis of CD was established by clinical, radiological, endoscopic, and histopathological findings.

Medical treatment of these patients consisted of prednisolone (less than 20 mg/day) and salicylazosulfapyridine (less than 3 g/day). One patient was receiving prednisolone, three patients were receiving prednisolone and salicylazosulfapyridine, and nine patients were receiving salicylazosulfapyridine alone. The disease involved the small bowel in three patients, the large bowel in four, and both small and large bowel in six.

Ten healthy volunteers of comparable age and sex distribution were employed as controls. The results of blood cell analysis for all controls were within the normal ranges.

Cell Isolation. Peripheral blood was drawn in sterile, disposable syringes with 10 units of heparin per milliliter of blood. After sedimentation of erythrocytes by dextran, mononuclear cells (MNCs) and polymorphonuclear cells (PMNs) were separated by centrifugation of leukocyte-rich plasma through a Ficoll-Conray mixture (Daiichi Chemical Co., Tokyo, Japan). Erythrocytes contaminating PMNs were removed by hypotonic cell lysis. PMNs were washed with Dulbecco's phosphate buffer solution (PBS) (Gibco Lab., Grand Island, New York) and suspended in minimal essential medium (MEM) (Gibco) supplemented with 25 mM HEPES (Sigma Chemical Co., St. Louis, Missouri). MNCs were also washed by Dulbecco's PBS and suspended in 25 mM HEPES MEM. The suspensions of both PMNs and MNCs were prepared to a concentration of 10^6 cells/ml for studies. Whole blood was diluted five times by 25 mM HEPES MEM as samples to measure.

Populations of purified lymphocytes were prepared by using carbonyl iron particles. Peripheral blood in which carbonyl iron was added was incubated and centrifuged through a Ficoll-Conray mixture to separate lymphocytes.

The percentages of monocyte populations in MNCs were calculated on smear films by peroxidase stain in phagocytes.

Preparation of Stimulants. Phorbol myristate acetate (PMA; Sigma) was dissolved in dimethylsulfoxide (Sigma) and mixed with MEM to a concentration of 10 μg/ml. Zymosan (Sigma) was opsonized by incubation with human sera and suspended in MEM. Both stimulants were stored at −70°C until the experiment.

Chemiluminescence Assay. A chemiluminescence (CL) analyzer Biolumat (Berthold, Germany) was used to measure CL activity generated by leukocytes.

Test vials containing 0.5 ml of the samples were initially warmed in an incubator at 37°C for 10 min. After addition of 0.02 mg luminol (Sigma) into the vials, CL activities generated by resting leukocytes were measured without the stimulants for 5 min. CL activities of stimulated leukocytes were measured continuously for 30–60 min during phagocytosis of opsonized zymosan or contact with PMA.

The CL activities counted for 2 sec were recorded consecutively on a TV monitor and saved in a computer for analysis. Statistical differences between subjects with Crohn's disease and controls were analyzed by Student's *t* test.

RESULTS

In order to clarify whether lymphocytes could generate CL, the CL activities of MNCs and those of purified lymphocytes were assessed. The CL activities generated by MNCs were reduced to low levels when MNCs were depleted of monocytes by removal with carbonyl iron particles (Figure 1). These data suggest that CL generated by MNCs was produced mostly by monocytes. The CL activities generated by MNCs and PMNs were in proportion to the numbers of cells (Figure 2). In calculating the comparative CL activities, data were expressed as CL counts/2 sec/10^4 phagocytes in whole blood, and CL counts/2 sec/polymorphonuclear cell and per monocyte in isolated cells.

The percentages of monocytes in MNCs from the patients with CD (33.3 ± 4.0%, $N = 13$, mean ± SEM) were significantly elevated ($P < 0.01$) when compared to those from the healthy volunteers (17.4 ± 1.5%, $N = 10$). The numbers of leukocytes (control 5720 ± 433/mm^3, $N = 10$, vs CD 5662 ± 621/mm^3, $N = 13$) and PMNs (control 3497 ± 362/mm^3, $N = 10$, vs CD 4146 ± 547/mm^3, $N = 13$), however, were not different between the two groups.

The peak levels of CL activity generated by diluted whole blood from the patients with CD were significantly higher ($P < 0.05$) than those from the healthy volunteers when it was stimulated by PMA (68.3 ± 11.5, $N = 13$, vs 37.7 ± 5.5 counts/2 sec/10^4 phagocytes, $N = 10$), but the former did not differ from the latter when it was incubated with opsonized zymosan (32.6 ± 5.2 vs 27.0 ± 4.7 counts/2 sec/10^4 phagocytes).

The peak levels of PMA- and opsonized zymosan-induced CL activity generated by monocytes from the patients with CD were 1.18 ± 0.27 and 1.23 ± 0.18 counts/2 sec/monocyte, respectively, and these from the healthy volunteers

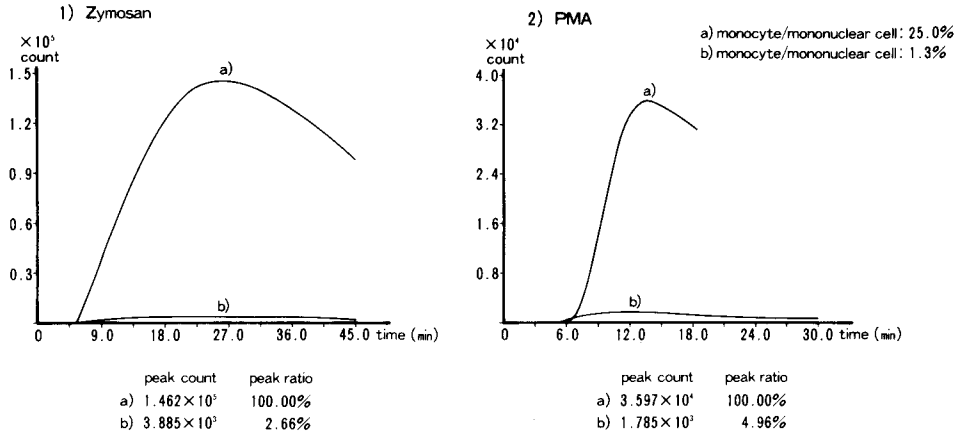

Fig 1. Generation of chemiluminescence by 5 x 10^5 mononuclear cells during incubation with opsonized zymosan or PMA: (a) monocyte-rich mononuclear cells before depletion by carbonyl iron; (b) monocyte-poor mononuclear cells after depletion by carbonyl iron.

were 0.43 ± 0.04 and 0.65 ± 0.04 counts/2 sec/monocyte, respectively.

Monocytes from the patients with CD generated significantly larger quantities of CL activity ($P < 0.01$) during incubation with both PMA and opsonized zymosan than those from the healthy volunteers (Figure 3).

In the case of PMNs, PMA-induced peak levels of CL activity (control 0.19 ± 0.02, $N = 10$, vs CD 0.19 ± 0.04 counts per cell, $N = 13$) and opsonized zymosan-induced peak levels of CL activity (control 0.31 ± 0.04, $N = 10$, vs CD 0.22 ± 0.04 counts per cell, $N = 13$) were not significantly different between the two groups.

The time taken to reach the CL peaks was significantly delayed only in PMA-induced CL generation by monocytes from the patients with CD when it was compared with those from the healthy volunteers ($P < 0.01$) (Figure 4).

DISCUSSION

Neutrophils increase the uptake of oxygen and generate active oxygen species when they are activated by various stimulants such as bacteria. It is known that these active oxygen species have a cytotoxicity even to normal tissues, as well as a bactericidal activity.

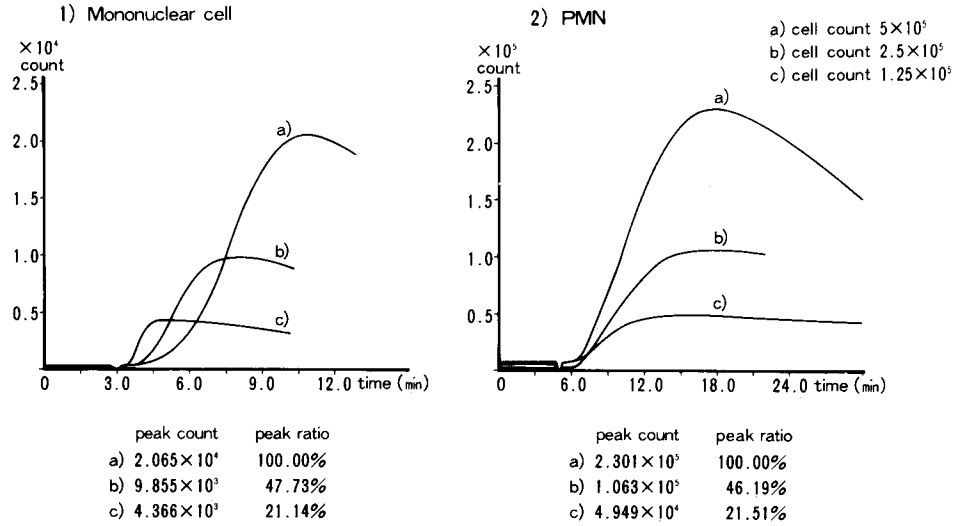

Fig 2. Relationship between cell counts of mononuclear cells or polymorphonuclear cells and generation of chemiluminescence during incubation with PMA.

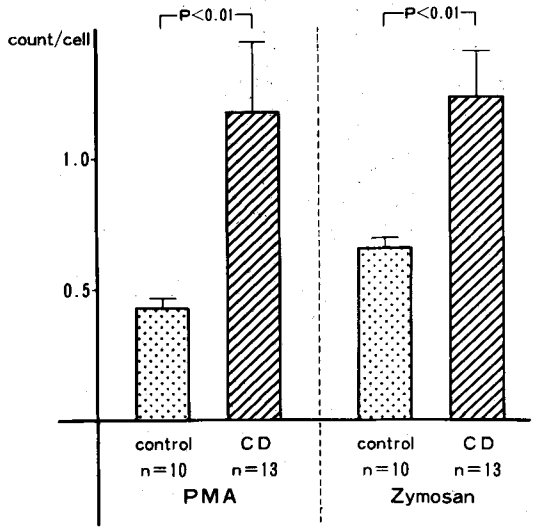

Fig 3. Peak chemiluminescence activities generated by monocytes on stimulation by PMA or opsonized zymosan. Mean values are expressed as CL counts/2 sec/monocyte; the bars indicate SEM.

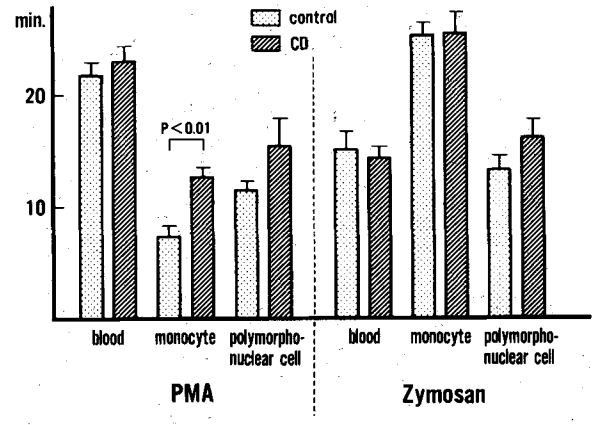

Fig 4. Time for diluated whole blood, monocytes, and polymorphonuclear cells to reach peak of chemiluminescence activities, which appeared during incubation with PMA or opsonized zymosan. The bars indicate SEM.

Chemiluminescence (CL) has been thought to occur coincident with the release of active oxygen species from neutrophils. A recent report indicated that monocytes, like neutrophils, released active oxygen species and generated CL during phagocytosis or on contact with surface perturbation (13). Our present study also demonstrated that the increase of CL generation was induced by phorbol myristate acetate (PMA) or opsonized zymosan, which increases the release of active oxygen species from both PMNs and monocytes. The data that lymphocytes were unable to generate CL are consistent with the report by Johnston et al (13). This implies that CL activity generated by mononuclear cells is derived from monocytes.

Our results suggest that monocytes from the patients with Crohn's disease (CD) release large amounts of active oxygen species during stimulation. In CD, epitheloid cell granuloma is a predominant and distinguishing feature of the chronic inflammation. This granulomatous reaction is assumed to be the result of abnormal phagocytosis. Epitheloid cell granulomas are formed by macrophages which stem from circulating monocytes. The present study may, therefore, provide a hypothesis that the excessive release of active oxygen species from monocytes and perhaps macrophages in CD may play an important role in formation of the intestinal lesions.

Histological findings of the lesions in CD resemble those found in chronic granulomatous disease (CGD) (1). Leukocytes from the patients with CGD are unable to produce active oxygen species (11). In our preliminary study, both monocytes and PMNs from patients with CGD are unable to generate PMA- and opsonized zymosan-induced CL. The function of monocytes in CD, therefore, is apparently different from that in CGD.

The function of PMNs is also different between CD and CGD because PMNs in CD are able to generate CL almost normally. Worsaae and coworkers reported a reduced and delayed staphylococcus-induced granulocyte CL response and normal nitroblue tetrazolium reduction test in CD (7). In our study, the zymosan-induced PMN CL response is slightly reduced and delayed in CD as compared with controls, but the results are not statistically significant. Recently, it was also demonstrated that neutrophils of untreated CD patients showed a deficient production of hydrogen peroxide whereas the production of superoxide anion was normal, and in treated CD patients this function of neutrophils recovered (14). The chemiluminescent response of neutrophils from patients with CD and ulcerative colitis was increased when compared to that of healthy subjects (8). Furthermore, previous investigations have shown an impaired, normal, or enhanced nitroblue tetrazolium reduction by neutrophils (6–8), and there are similar results about phagocytosis in CD (5, 6, 9, 10). These differences may be explained by differences in experimental design; however, it is also hypothesized that some factors which effect a function of neutrophils may be important in explaining these discrepancies in neutrophil functions in CD.

In studies on mobilization using a skin-window technique, defective neutrophil function in CD was found, whereas neutrophil chemotaxis *in vitro* was normal and cellular dysfunction could not be demonstrated (4–6). In this phenomenon, the presence of serum inhibitors of chemotaxis was suggested (6, 15). Our study also showed the discrepancy in that whole blood from the patients with CD generated larger quantities of PMA-induced CL than that from controls, in contrast to the results of separated PMNs. The discrepancy in these results may also be a result of serum factors. It has been reported that chemotactic factors were inactivated by the myeloperoxidase–hydrogen peroxide–halide system (16). An immunosuppressive factor has been recognized in the sera of the patients with CD (17). Monocytes are necessary for an immune response of lymphocytes; however, excessive active oxygen species released from monocytes inhibit mitogen stimulation of lymphocytes (18). There is a possibility that the increased release of active oxygen species from monocytes in CD may modulate the mitogen stimulation of lymphocytes and explain in part one of the immunosuppressive factors.

The present study has demonstrated excessive production of active oxygen species from monocytes in CD. However, further studies on the mechanism of this phenomenon and the serum factors that affect leukocyte function are necessary. More recently, it was reported that activated alveolar macrophages in CD may play a part in the transient alterations of pulmonary function through a generation of active oxygen species (19). Studies on intestinal macrophages probably will be more significant in the pathogenesis of CD than circulating monocytes.

REFERENCES

1. Ament ME, Ochs HD: Gastrointestinal manifestations of chronic granulomatous disease. N Engl J Med 288:382–387, 1973
2. Holmes B, Dage AR, Good RA: Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. J Clin Invest 46:1422–1432, 1967
3. Baehner RL, Nathan DG, Karnovsky ML: Correction of metabolic deficiencies in the leukocytes of patients with chronic granulomatous disease. J Clin Invest 49:865–870, 1970
4. Segal AW, Loewi G: Neutrophil dysfunction in Crohn's disease. Lancet 1:219–221, 1976
5. O'Morain C, Segal AA, Walker D, Levi AJ: Abnormalities of neutrophil function do not cause the migration defect in Crohn's disease. Gut 22:817–822, 1981
6. Wandall JH, Binder V: Leucocyte function in Crohn's disease. Gut 23:173–180, 1982
7. Worsaae N, Staehr Johansen K, Christensen KC: Impaired *in vitro* function of neutrophils in Crohn's disease. Scand J Gastroenterol 17:91–97, 1982
8. Ward M, Eastwood MA: The nitroblue tetrazolium test in Crohn's disease and ulcerative colitis. Digestion 14:179–183, 1976
9. Krause U, Michaelsson G, Juhlin L: Skin reactivity and phagocytic function of neutrophil leucocytes in Crohn's disease and ulcerative colitis. Scand J Gastroenterol 13:71–75, 1978
10. Ward M: Phagocytic function in Crohn's disease. Z Gastroenterol 17(suppl):116–124, 1979
11. Curnutte JT, Whitten DM, Babior BM: Defective superoxide production by granulocytes from patients with chronic granulomatous disease. N Engl J Med 290:593–597, 1974
12. Croften RW, Martina MC, Diesselhoff-Den Dulk MMC, van Furth R: The origins, kinetics and characteristics of the Kupffer cells in the normal steady state. J Exp Med 148:12-17, 1978
13. Johnston RB, Lehmeyer JE, Guthrie LA: Generation of superoxide anion and chemiluminescence by human monocytes during phagocytosis and on contact with surface bound immunoglobulin G. J Exp Med 143:1551–1556, 1976
14. Verspaget HW, Mieremet-Ooms MAC, Weterman IT, Pena AS: Partial defect of neutrophil oxidative metabolism in Crohn's disease. Gut 25:849–853, 1984
15. Rhodes JM, Jewell DP: Serum inhibitors of leucocyte chemotaxis in Crohn's disease and ulcerative colitis. Gut 20:A911, 1979
16. Clark RA, Klebanoff SJ: Chemotactic factor inactivation by the myeloperoxidase–hydrogen peroxide–halide system. An inflammatory control mechanism. J Clin Invest 64:913–919, 1979
17. Watanabe M, Tsuru S, Zinnaka Y, Yoshida T, Aiso S, Hibi T, Asakura H, Tsuchiya M: Demonstration and characterization of immunosuppressive factors in sera from patients with Crohn's disease. J Clin Lab Immunol 17:13–19, 1985
18. Metzger ZV, Hoffeld JT, Oppenheim JJ: Macrophage-mediated suppression. 1. Evidence for participation of both hydrogen peroxide and prostaglandins in suppression of murine lymphocyte proliferation. J Immunol 124:983–989, 1980
19. Wallaert B, Aerts C, Bonniere P, Cortet A, Tonnel AB, Paris JC, Voisin C: Superoxide anion generation by alveolar macrophages in Crohn's disease. N Engl J Med 312:444–445, 1985