Interleukin 2 and interferon- γ augment anticolon antibody dependent cellular cytotoxicity in ulcerative colitis

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Abstract

In vitro effects of cytokines and therapeutic drugs on antibody dependent cellular cytotoxicity (ADCC) mediated by anticolon antibody were investigated in serum samples from patients with ulcerative colitis. A ^{51}Cr release assay was used to examine ADCC activity with the colon cancer cell line, colo 205, as the target and peripheral blood mononuclear cells as the effector. High ADCC activity was shown in 13 of 32 (41%) patients with ulcerative colitis. This ADCC activity was inhibited by protein A treatment of the serum samples. Interleukin 2 (IL2) activated effector cells could enhance ADCC activity, but interferon- γ (IFN- γ) or tumour necrosis factor- α (TNF- α) had no effect on the cytotoxic activity of effector cells. Treatment of target cells with IFN- γ increased the vulnerability of these cells to ADCC with a large increase of intercellular adhesion molecule-1 (ICAM-1) expression on their surface. Monoclonal antibodies to ICAM-1 inhibited this IFN- γ enhanced ADCC activity. Interestingly, prednisolone (PSL) reduced ADCC activity, but sulphasalazine (SASP) or 5-aminosalicylic acid (5-ASA) did not. These results suggest that IL2 and IFN- γ could enhance colonic epithelial cell injury mediated by the ADCC mechanism in ulcerative colitis and that ADCC enhanced by cytokines is restored by PSL treatment.

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Immunological abnormalities are generally accepted to be involved in the pathogenesis of ulcerative colitis. T cell mediated immunity and microcirculatory disturbance may participate in the induction of colonic inflammation. Circulating anticolon antibodies have been found in serum samples from patients with ulcerative colitis.¹⁻³ Whether or not these autoantibodies are directly related to the pathogenesis of this disease remains controversial.^{4,5} There are some studies indicating that these autoantibodies may contribute to lysis of colon epithelial cells through antibody dependent cellular cytotoxicity (ADCC) mechanisms.⁶⁻⁹ We have previously reported that these autoantibodies are synthesised by mucosal or peripheral blood lymphocytes from patients with ulcerative colitis through the use of the Epstein Barr virus (EBV) transformation method.¹⁰

There is no doubt that cytokines can contribute to the pathophysiology of the diseases in some chronic inflammatory states and autoimmune conditions. Reports of in vitro produc-

tion of cytokines by peripheral or mucosal mononuclear cells in inflammatory bowel disease, however, conflict.¹¹⁻¹⁶ It is postulated that cytokines may affect colonic inflammation, but it still remains unclear how they contribute to the exacerbation of the inflammation. As cytokines modulate cytotoxic activities of various killer cells,¹⁷⁻²⁰ perhaps ADCC mediated by anticolon antibody (anticolon ADCC) is as well modulated in vivo by the cytokines synthesised locally in the colonic mucosa of ulcerative colitis.

It has been considered that therapeutic drugs such as sulphasalazine (SASP), 5-aminosalicylic acid (5-ASA), and prednisolone (PSL) may reduce the accelerated cytotoxicity. In this study, we first investigated the effects of cytokines on anticolon ADCC activity and then examined the effects of therapeutic drugs on anticolon ADCC activity, natural killer activity, and lymphokine activated killer activity.

Patients and methods

SERUM SAMPLES

Ulcerative colitis was diagnosed on the basis of typical, clinical, radiographic, and pathological features. Serum samples were taken from 32 patients with ulcerative colitis (19 males and 13 females). The mean age of the patients with ulcerative colitis was 36 (range 16-55) years. The colitis was active in 17 patients and inactive in 15. The serum samples from nine patients with Crohn's disease, six patients with colonic diverticulitis, and seven patients with colonic cancer were also tested. Control serum samples were taken from 31 healthy hospital workers. The sex and age distribution of the healthy control group was comparable with that of the patient group. Serum samples were stored at -80°C without preservatives and centrifuged at $105\,000\text{ g}$ for 90 minutes before use. The activity of anticolon antibody in the serum samples was detected by enzyme linked immunosorbent assay (ELISA) with colon epithelial cells as reported previously.¹⁰

MONONUCLEAR CELL PREPARATION

Heparinised blood was taken from apparently healthy volunteers for use as effector cells for cytotoxicity. Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll-Hypaque density gradient through centrifugation. After being washed with phosphate buffered saline (PBS), the PBMCs from healthy controls were

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used for the ADCC assay as effector cells. To elucidate the characteristics of effector cells, PBMCs were separated into monocytes and lymphocytes by adherence onto Petri dishes. The resulting population of adherent cells, subsequently designated monocytes, was more than 95% esterase positive and showed the morphological characteristics of monocytes as determined by Giemsa stained preparations.

ADCC ASSAY

A ^{51}Cr release assay was used to determine ADCC activity. The human cancer cells from the cell line colo 205 that expressed colonic antigens detected by anticolon antibody positive serum was used as the target cells and PBMCs from healthy controls were used as effector cells. These effector cells were preincubated with or without 1–100 U/ml of recombinant interleukin 2 (IL2) (Shionogi Pharm, Osaka, Japan) or 1–100 U/ml of recombinant interferon- γ (IFN- γ) (Shionogi Pharm, Osaka, Japan) for one day. The target colo 205 cells were preincubated with or without 100 U/ml of IFN- γ for two days. Recombinant human tumour necrosis factor- α (TNF- α) (Boehringer Mannheim, Germany) (0.1–100 ng/ml) was added to effector cells and incubated for one day. Target cells were treated with TNF- α for two days. After being washed with medium, the target cells were suspended in 1 ml of medium containing 100 mCi $\text{Na}_2^{51}\text{CrO}_4$ (Daiichi Radioisotope, Tokyo, Japan) and incubated for 45 minutes at 37°C. The labelled cells were washed with medium and resuspended at a concentration of 1×10^5 cells/ml. A 100 μl suspension of ^{51}Cr labelled target cells were incubated with 50 μl of 1:10 diluted serum from the patients with ulcerative colitis and 50 μl of effector cells (1:50 ratio of effector to target) for four hours at 37°C. Culture of target cells and effector cells without serum was used as the control (control culture). All cultures were performed in triplicate. At the end of the incubation, supernatant was collected from each well and the radioactivity was measured with a γ -counter. Maximum possible ^{51}Cr release was determined by three freeze thaw cycles. ADCC activity was calculated according to the following formula, in which spontaneous cytotoxicity was reduced:

% cytotoxicity =

$$\frac{(\text{experimental release} - \text{release of control culture})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

Staphylococcus aureus protein A (Sigma, St Louis, MO) was used for the inhibition assay. This compound is known to bind to the Fc portion of IgG and to block the interaction between Fc of IgG and Fc receptor on effector cells.²¹ The serum samples were incubated with 30 $\mu\text{g}/\text{ml}$ of *staphylococcus aureus* protein A for 10 minutes and then centrifuged.

Monoclonal antibody to intercellular adhesion molecule-1 (ICAM-1) (Immunotech SA, Cedex, France, human leucocyte antigen (HLA) A,B,C (BB 7-7, ATCC, Rockville, MD), and HLA-DR (L243, ATCC, Rockville, MD) was used at the concentration of 2 $\mu\text{g}/\text{ml}$ to inhibit ADCC activity.

NATURAL KILLER ASSAY

K-562 cells ($1 \times 10^5/\text{ml}$) labelled with ^{51}Cr were used as target cells. After the target cells were incubated with PBMCs ($2 \times 10^6/\text{ml}$) for four hours, ^{51}Cr activity in the supernatant was measured. The results were expressed in % cytotoxicity as follows:

% cytotoxicity =

$$\frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

LYMPHOKINE ACTIVATED KILLER ASSAY

^{51}Cr labelled Daudi cells ($1 \times 10^5/\text{ml}$) were used as target cells; PBMCs ($2 \times 10^6/\text{ml}$) were incubated with 1000 U of IL2 for two days and then cultured with the ^{51}Cr labelled target cells for four hours. The results were expressed in % cytotoxicity according to the formula given for the natural killer assay.

EFFECTS OF DRUGS ON CYTOTOXICITY ACTIVITIES

To examine the effects of PSL, SASP, and 5-ASA on ADCC activity as well as on natural and lymphokine activated killer activity, those drugs were added at various concentrations to the cultures in all three kinds of assays already described.

IMMUNOFLUORESCENCE STAINING AND FLOW CYTOMETRY ANALYSIS

One million cells were incubated at 4°C for 30 minutes with monoclonal antibody to ICAM-1. After washing, the cells were stained with fluorescence isothiocyanate conjugated rat anti-mouse Ig monoclonal antibody (Dako, Santa Barbara, CA, USA) at 4°C for 30 minutes. For the staining of HLA-A,B,C and HLA-DR, FITC conjugated anti-HLA-A,B,C (W6/32, Sera-Labo, Sussex, UK) and anti-HLA-DR (L243, Becton Dickinson, Mountain View, CA, USA) were used. After two washes, the stained cells were assayed for fluorescence intensity on a FACScan cell sorter (Becton Dickinson, Mountain View, CA, USA).

Results

ADCC ACTIVITY IN THE SERUM SAMPLES FROM PATIENTS WITH ULCERATIVE COLITIS

The mean ADCC activity of the serum samples from 32 patients with ulcerative colitis was 9.6 (SD 9.9)% and that from normal controls was 2.8 (2.5)%. The difference was significant ($p < 0.001$) (Fig 1). Anticolon activities measured by enzyme linked immunosorbent assay (ELISA) were 0.918 (0.281) (optical densities) in serum samples from patients with ulcerative colitis and 0.466 (0.144) in control samples. Simple regression showed a significant correlation between the values in ELISA and ADCC activity ($r = 0.615$, $p < 0.05$). In 17 patients with active ulcerative colitis, the mean cytotoxicity was 13.5 (SD 12.0)%, which was significantly higher than that of healthy controls. By contrast, the cytotoxicity of 15 patients in the inactive stage was 4.8 (3.4)%, which was not significantly

Figure 1: Cytotoxicity (%) against colon cancer cell (colo 205) of the serum samples of normal controls and patients with ulcerative colitis, Crohn's disease, colon diverticulitis, or colon cancer (spontaneous cytotoxicity without serum was subtracted). Cytotoxicity of the serum samples of patients with ulcerative colitis was significantly higher than that of normal controls.

Figure 2: Effects of IL2, TNF- α , and IFN- γ on anticolon ADCC activity. Peripheral blood mononuclear cells (PBMCs) were incubated with IL2, TNF- α , and IFN- γ , and ADCC activity was examined. ADCC activity was greatly enhanced by IL2 but was not influenced by TNF- α or IFN- γ .

Figure 3: Effects of IFN- γ on ADCC activity and colon antigen expression. Target colo 205 cells were incubated with 100 U/ml of IFN- γ for two days. Colon antigen expression was examined by ELISA with an anticolon antibody positive serum. Activity of ADCC was significantly increased by IFN- γ treatment, whereas the antigen expression was not altered by IFN- γ treatment.

different from that of normal controls. A percentage cytotoxicity above the normal range (mean (2 SD) in normal controls) was found in serum samples from 13 of 32 (41%) patients with ulcerative colitis. The percentage cytotoxicities of nine patients with Crohn's disease and six patients with colonic diverticulitis were 4.44 (1.82)% and 3.94 (2.04)%. These were not significantly higher than the percentage cytotoxicity of normal controls. In serum samples from seven patients with colon cancer, the percentage cytotoxicity was 2.7 (1.9)%, which was not significantly different from that of serum samples from normal controls. The serum samples from patients with high cytotoxic activity were used for the subsequent ADCC assay.

To confirm that the increased cytotoxicity was due to the interactions between the Fc portion of IgG and the Fc receptor on the effector cells, *Staphylococcus aureus* protein A was added to the ADCC assay. It was found that *Staphylococcus aureus* protein A had a pronounced inhibition on ADCC (mean of 71% with a range of 51% to 92% inhibition).

EFFECT OF IL2 ON ADCC ACTIVITY

The % cytotoxicity of the six serum samples from patients with ulcerative colitis was 11.2 (4.3)% when untreated effector cells were used (Fig 2(A)). Preincubation of the effector cells with 1 U/ml, 10 U/ml, or 100 U/ml of IL2 enhanced ADCC activity in the serum samples from patients with ulcerative colitis (19.1 (2.0)%, 37.3 (3.0)%, and 40.2 (2.9)%). This enhanced cytotoxicity was also inhibited by treatment with *Staphylococcus aureus* protein A.

EFFECT OF TNF- α ON ADCC ACTIVITY

The effector cells were incubated with or without various concentrations of TNF- α , and their ADCC activity was tested. The treatment of effector cells with TNF- α did not alter ADCC activity in six serum samples from the patients (Fig 2(B)). In the following experiments, five or six serum samples were used unless indicated otherwise. When the target cells were treated with TNF- α at a concentration of 1 to 100 ng/ml, ADCC activity against TNF- α treated cells was not significantly different from that against untreated target cells (data not shown).

EFFECT OF IFN- γ ON ADCC ACTIVITY

The effector cells were preincubated with 1 to 100 U/ml IFN- γ , and the resulting ADCC activities were compared (Fig 2(C)). Treatment of effector cells with IFN- γ did not enhance ADCC activity. When the target cells were preincubated with 100 U/ml of IFN- γ for two days, the ADCC activity of the serum samples from six patients with ulcerative colitis was 23.9 (2.0)%. It was significantly increased in comparison with the % cytotoxicity of the same serum samples against untreated target cells (11.6 (2.6)%) (Fig 3(A)).

The colon antigen expression detected by anticolon antibody positive serum samples was examined in IFN- γ treated and untreated target

Figure 4: Effects of TNF- α and IFN- γ on ICAM-1, HLA-DR, and HLA-A,B,C expression of colo 205 cells. Colo 205 cells were incubated with 10 ng/ml of TNF- α or 100 U/ml of IFN- γ for two days. ICAM-1 was not found on the surface of untreated cells (-) and slightly induced on TNF- α treated cells. Almost all IFN- γ treated cells expressed ICAM-1 on their surface. TNF- α did not induce HLA-A,B,C expression, but increased HLA-DR expression slightly. IFN- γ induced HLA-DR expression and increased HLA-A,B,C expression.

cells. The activities of anticolon antibody detected by ELISA expressed as optical density were compared. The mean reactivity of serum samples from five patients with ulcerative colitis was 0.766 (0.089) (optical density units) when the cells were untreated. When the cells were treated with IFN- γ , the reactivity was 0.832 (0.067) (NS, Fig 3(B)).

In the next experiment, the expression of ICAM-1, HLA-A,B,C or HLA-DR was examined on the surface of TNF- α (10 ng/ml) or

IFN- γ (100 U/ml) treated and untreated target cells. None of the untreated cells expressed ICAM-1, and TNF- α treatment induced a slight ICAM-1 expression on the cell surface. By contrast, almost all of the IFN- γ treated cells expressed this molecule (Fig 4). Furthermore, monoclonal antibody to ICAM-1 significantly inhibited IFN- γ enhanced ADCC activity (cytotoxicity: 23.2 (3.6)% without the monoclonal antibody and 12.4 (0.9)% with it). The ADCC activity without IFN- γ was unaltered by monoclonal antibody to ICAM-1 (cytotoxicity: 14.5 (3.1)% without the monoclonal antibody, 12.4 (2.2)% with it). The HLA-DR was not expressed on untreated and TNF- α treated colo 205. Treatment with IFN- γ profoundly induced the expression of HLA-DR on the surface of colo 205. The expression of HLA-A,B,C was slightly increased by TNF- α and considerably increased by IFN- γ (Fig 4). Monoclonal antibody, however, to either HLA-DR or HLA-A,B,C did not inhibit IFN- γ enhanced ADCC activity (cytotoxicity: 22.1 (4.5)% without monoclonal antibody, 20.1 (3.3)% with monoclonal antibody to HLA-DR, 22.8 (6.4)% with monoclonal antibody to HLA-A,B,C).

Figure 5: Effects of PSL, SASP, and 5-ASA on ADCC, lymphokine activated killer (LAK), and natural killer (NK) activity. PSL inhibited ADCC activity, but SASP or 5-ASA did not. LAK activity was profoundly inhibited by PSL but not by SASP or 5-ASA. PSL inhibited NK activity, and SASP at high concentrations (50 μ g/ml) showed slight inhibition on NK activity.

EFFECTS OF THERAPEUTIC DRUGS ON ADCC, NATURAL AND LYMPHOKINE ACTIVATED KILLER ACTIVITY

The addition of PSL to the ADCC assay culture with the serum samples from patients with ulcerative colitis decreased percentage cytotoxicity. In the presence of PSL, there was significant reduction in lymphokine activated killer activity as well (Fig 5). Natural killer activity, however, was not greatly influenced by the addition of PSL. The addition of 0.5 to 50 μ g/ml SASP to the three types of cytotoxicity assay did not alter percentage cytotoxicity. The 5-ASA did not affect any cytotoxic activity significantly whereas ADCC and lymphokine

activated killer activity were slightly decreased at a high 5-ASA concentration.

Discussion

Various kinds of cytokines may be involved in causing the inflammation of colonic mucosa in ulcerative colitis. The production of IL1, IL2, TNF- α , and IFN- γ is probably increased in patients with inflammatory bowel diseases.¹¹⁻¹⁴ These cytokines may affect the functions of immunocompetent cells to induce colonic injury or induce the lysis of colonic epithelial cells directly.²² It is well established that cytokines including IFN- α , IFN- β , IFN- γ , IL2, TNF- α , and the combination of these can enhance the cytotoxic activity of T cells, natural killer cells, and monocytes in vitro.^{17-20 23 24} Activity of ADCC is also enhanced by various cytokines including IFN- γ , IL2, and the combination of IL2 and TNF.²⁵⁻²⁷ Previous reports, however, indicate that the effects of cytokines were tested in the monoclonal antibody mediated cellular cytotoxicity (monoclonal ADCC) system. The effects of cytokines on ADCC mediated by autoantibody in human serum, though, have never been tested. The synthesis of cytokines such as IL2 and TNF- α is increased in the colonic mucosa of inflammatory bowel disease, whereas some reports postulate against the increased production of IFN- γ .^{15 16} It is assumed that these increased cytokines can induce ADCC in colonic inflammation such as ulcerative colitis as well as in some malignancies.

In our study, IL2 activated effector cells were found to exert considerable ADCC activity, but IFN- γ treated effector cells did not. Taken together with the previous study on the IL2 induced ADCC in the murine system,²⁵⁻²⁷ the increase in Fc receptor positive cell populations by treatment with IL2 may have enhanced the anticolon ADCC activity in our study.

Unexpectedly, treatment of effector cells or target cells with TNF- α did not affect ADCC activity. It has recently been suggested that the synthesis of TNF- α is greatly increased in inflammatory bowel disease and that TNF- α could enhance the functions of natural killer cells,¹⁸ lymphokine activated killer cells, and polymorphonuclear neutrophils.²⁸ The results in our study, however, indicate that TNF- α does not participate in anticolon ADCC mechanisms to kill colonic epithelial cells.

Target cells treated with IFN- γ were more vulnerable to anticolon ADCC. The increased expression of the colon antigen on target cells by IFN- γ treatment might have been expected to enhance anticolon ADCC activity, but there was no significant difference in the colon antigen expression tested by anticolon antibody positive serum samples between the IFN- γ treated and untreated target cells. One of the explanations for this unexpected result is that the colon specific antigens detected by ELISA and those detected by this ADCC assay may be different, and those detected by ADCC might be increased by treatment with IFN- γ . Alternatively, some surface antigens relating to ADCC mechanisms may be changed by the treatment. Recent studies²⁹⁻³¹ have proposed that the molecules

mediating cellular adhesion such as LFA-1 and ICAM-1 are important in the cytotoxic activity of monocytes and T cells. Thus we examined the role of ICAM-1 in the IFN- γ induced vulnerability of target cells. We found that anti-ICAM-1 antibody could block the enhancement of anticolon ADCC through IFN- γ treatment of target cells, on which the ICAM-1 expression was remarkably induced. This result clearly indicates that the enhanced ICAM-1 expression on target cells may induce the vulnerability of the target cells to anticolon ADCC.

We next examined the influence of corticosteroid, SASP or 5-ASA on anticolon ADCC. It has been reported that spontaneous cell mediated cytotoxicity brought on by mononuclear cells can be inhibited by SASP but not by corticosteroid, and that ADCC is not influenced by SASP or corticosteroid.³² Gibson and Jewell have reported that SASP produces an inhibitory effect on natural killer activity.³³ In our study, corticosteroid was found to result in anticolon ADCC and lymphokine activated killer activity. These results show a considerable disparity with those reported by MacDermott *et al.*,³² and the reasons for this are not clear. The different target cells and the assay systems used might explain the discrepancy.

In conclusion, the in vitro effects of these cytokines on ADCC activity suggest that locally produced cytokines exacerbate the lysis of colonic epithelium through the enhancement of ADCC mechanisms as well as by direct effects of these cytokines. The treatment with corticosteroid is suggested to be effective through the inhibition of anticolon ADCC in the colonic mucosa. As anticolon ADCC in patients with ulcerative colitis is one of the various immunological abnormalities that contribute to the colonic inflammation, it is accordingly of considerable value to find drugs capable of inhibiting anticolon ADCC.

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