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In vitro production of myeloperoxidase anti-neutrophil cytoplasmic antibody and establishment of Th1-type T cell lines from peripheral blood lymphocytes of patients

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ABSTRACT

Objective. *To investigate the pathogenic role of T cells in the development of anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis.*

Methods. *Peripheral blood lymphocytes (PBL) were isolated from myeloperoxidase anti-neutrophil cytoplasmic antibody (MPO-ANCA) associated vasculitis patients and cultured in medium. The production of MPO-ANCA in the medium of PBL stimulated with Concanavalin-A (Con-A), with or without cyclosporin (CyA), was measured by enzyme-linked immunosorbent assay (ELISA) on MPO coated plates. RNA isolated from PBMC of one patient was used for polymerase chain reaction (PCR) and single stranded conformational polymorphism (SSCP) studies, and MPO-specific T cell lines (TCL) were established by antigen stimulation techniques.*

Results. *PBL of patients with MPO-ANCA-associated vasculitis produced MPO-ANCA following Con-A stimulation, and this effect was inhibited by treatment with cyclosporin A (CyA) or elimination of CD4 cells. PCR-SSCP showed autoantigen-reactive oligoclonal T-cell accumulation in PBMC of one of these patients. We established MPO-specific TCL which secreted interferon-γ (IFN-γ), but not interleukin-4 (IL-4); all TCL were CD4 positive, CD8 negative, and HLA-DR restricted.*

Conclusions. *Our results suggest that Th1-type T cells may mediate MPO-ANCA production, and may play a role in the onset of MPO-ANCA vasculitis.*

Introduction

Antineutrophil cytoplasmic antibodies (ANCA) are important markers for small vessel vasculitis and pauci-immune glomerulonephritis (1, 2). However, the onset mechanism of ANCA associated vasculitis remains unclear. Prolonged or unusual presentation of ANCA antigens to the immune system in the context of a specific genetic background may lead to the formation of autoantibodies, but the processes involved are not fully understood. The aim of this study was to investigate the immune response to ANCA antigens using T cell-mediated immune reactions, and to determine whether the Th1 or Th2 T cell subtypes are primarily responsible for providing the T cell help to B cells producing ANCA

Patients and methods

Patients

Three MPO-ANCA-positive patients (2 women, mean age 57.3 ± 8.9 years, and 1 man, age 67.0 years and 3 healthy donors (2 women, mean age 57.0 years, and 1 man, age 65 years) participated in this study. All patients had rapidly progressive crescentic glomerulonephritis, one had pulmonary hemorrhage, one had pleuritis, and one had interstitial pneumonitis. MPO-ANCA levels ranged from 320 to 1300 ELISA units.

Cell preparation

PBL were separated using Ficoll/Hypaque and G-10 columun. Purification of T cell subpopulations was performed using CD4 monoclonal antibodies and complement. (Behringwerke) (3). Briefly, 1×10^7 purified T cells were incubated with 100 μl anti-CD4 antibody on ice for 1 hr. Subsequently, 25 μl complement (Behringwerke AG, Germany) was added and the mixture was incubated for 1 hr at 37°C. Cells were washed and the procedure was repeated.

In vitro *MPO-ANCA assay*

MPO-ANCA was determined using ELISPOT assay as previously described (4); briefly the method was as follows. PBL or CD4-depleted PBL were taken into tissue culture plates coated with MPO antigen (Nissho), and cultured for 4 days with several mitogens (Con-A, LPS, TNF-α and IL-1β) and cyclosporin (CyA) at various concentrations. The bound antibodies were detected by adding peroxidase-labeled anti-human IgG, followed by an addition of substrate. The resultant color was measured photometrically at 405 nm using an ELISA plate reader. Cell proliferation was measured by standard tritium-labeled thymidine ([^{3}H]TdR) incorporation, as described (5).

IFN-γ and IL-4 measurements

IFN-γ and IL-4 were assayed by ELISA kits (MBL, Nagoya, Japan). We

measured the cytokines on days 2, 3, and 4. The results were measured photometrically at 405 nm using an ELISA plate reader.

PCR and the SSCP analysis

The SSCP method used to study the T cell antigen receptor (TCR) was performed as described previously (6, 7). Briefly, total RNA was isolated from the PBMC of one patient and converted to cDNA using reverse transcriptase (BRL). The cDNA reaction mixture was then mixed with each primer set (a Vβ primer and a biotinylated constant region β-chain primer). PCR was performed after addition of dNTPs and Taq DNA polymerase (Promega) for 35 cycles (94°C for 1.5 min, 60°C for 2 min, and 72°C for 3 min) in a Perkin-Elmer Cetus thermocycler. Amplified DNA samples were electrophoresed in a non-denaturing 5% polyacrylamide gel. After electrophoresis, DNA was transferred to Immobilon-S membranes (Millipore) and visualized by subsequent incubations with streptavidin, conjugated alkaline phosphatase, and a chemiluminescent substrate (Plex Luminescence kit, Millipore).

Establishment of MPO-specific T-lymphocyte lines

TCL were generated from PBL by antigen restimulation techniques, as described (8). Briefly, MPO was added to a suspension of the PBL of one patient at a final concentration of 30 μg/ml, then 3 U of IL-2 was added every 3-4 days. Autologous irradiated (40 Gy) peripheral blood leukocytes were added to subcultures, after which MPO was added every 14 days. TCL were cultured with β-lactoglobulin, native MPO (a gift from Nissho, Japan), anti HLA-DR antibodies, anti HLA-DQ antibodies, or MPO alone. Cultures were incubated for 96 hrs at 37°C in a humidified 5% CO_2 atmosphere. Six hours prior to the termination of the incubation period, 0.5 μCi of [^{3}H]TdR

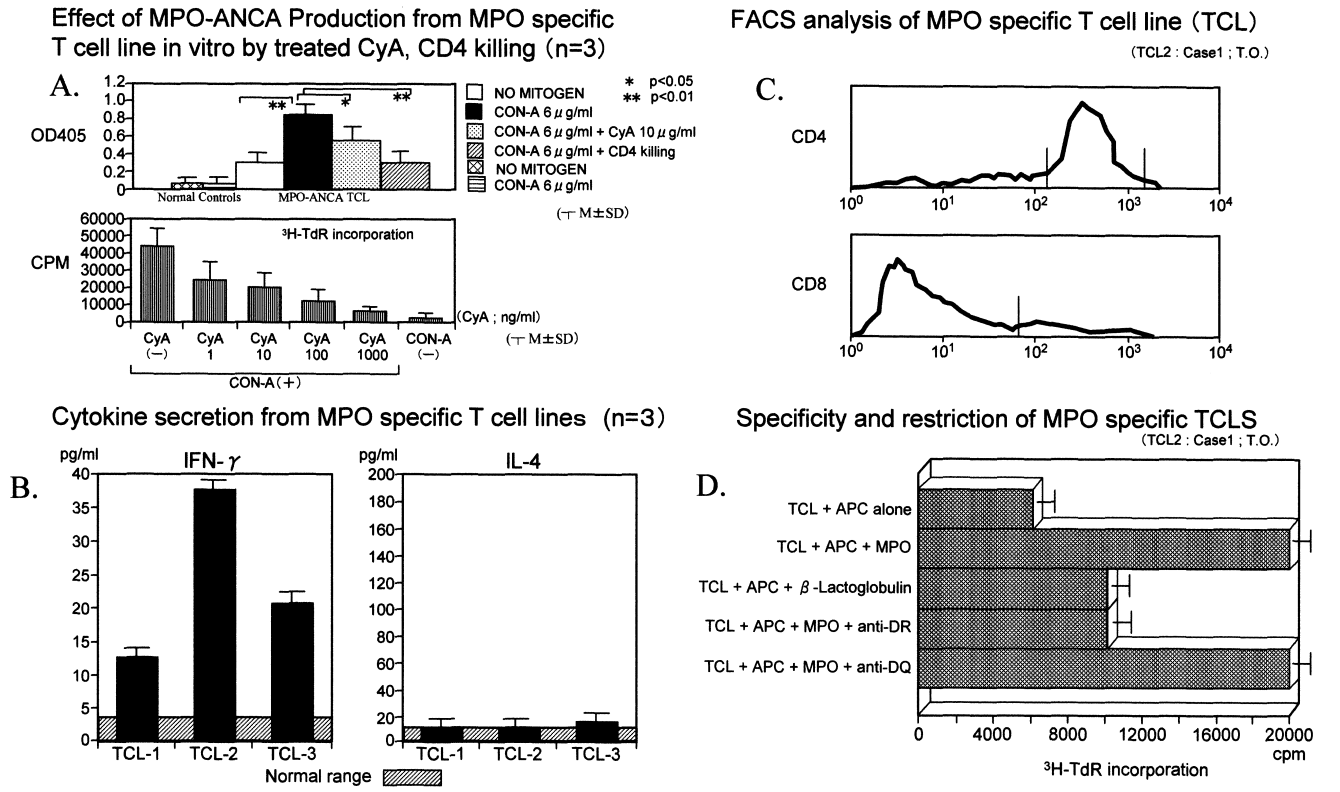

Fig. 1. (**A**) Dose-dependent inhibition of MPO-ANCA production by PBL from MPO-ANCA associated vasculitis patients by CyA treatment and CD4 depletion. **Upper panel:** 1 x 10^6 PBL from patients and normal controls were cultured in MPO antigen coated plates in medium alone and in the presence of 6 μg/ml Con-A plus 10 ng/ml CyA, or in the presence of 6 μg/ml Con-A without CD4 T cells. (*$p < 0.05$; **$p < 0.01$). **Lower panel:** 1 x 10^6 PBL from patients were cultured in MPO antigen coated plates in medium alone, or the presence of 6 μg/ml of Con-A plus CyA at 0, 1, 10, 100, or 1000 ng/ml, and the incorporation of ^{3}H-TdR was measured. Values are expressed as the mean of triplicate wells.
(**B**) TCLs established from 3 MPO-ANCA associated vasculitis secreted IFN-γ but not IL-4 (1, 2, 3). 1 x 10^6 PBL from patients or healthy controls were cultured for 96 hrs in medium alone or with 6 μg/ml of Con-A. IL-4 and IFN-γ secreted into the culture supernatants were measured by specific ELISAs for each cytokine. Data shown are representative of 3 to 6 experiments, depending on the cytokine.
(**C**) CD4 expression of T cell lines. A representative CD4 T cell line TCL1 was stained with FITC-labeled anti-CD4 or anti-CD8 and analyzed by FACScan.
(**D**) HLA class II restriction of a MPO specific TCL. 1 x 10^6 cells of an established TCL were cultured for 96 hr with antigen presenting cells (APC) only, or APC plus β-Lactoglobulin or MPO in the presence of anti-DR or anti-DQ antibodies and [^{3}H]thymidine incorporation was measured. Results represent mean ± SD, n = 5.

was added to each well. Cells were then harvested, and incorporated radioactivity was measured by scintillation counting.

Statistical analysis

Statistical significance of measurements was performed by Student's t-test. A value of $P < 0.05$ was considered significant.

Results

Production of MPO-ANCA with Con-A

In order to investigate lymphocyte ANCA production, we examined the effects of several mitogens (Con-A, LPS, IL-1β, TNF-α, phytohemagglutinin, pokeweed mitogen, and) on PBL from patients with MPO-ANCA-associated vasculitis. MPO-ANCA was produced only by Con-A primed PBL of vasculitis patients but was not produced by PBL of healthy controls. PBL viability was not adversely affected at Con-A concentration of 6 μg/ml. Next, we studied the inhibitory effect of CyA and CD4 depletion. Results indicate that CyA partially inhibited, and CD4 depletion completely inhibited antibody production by PBL. PBL from MPO-ANCA vasculitis patients were able to proliferate with Con-A alone, and the inhibition of proliferation of PBL stimulated with Con-A in patients with MPO-ANCA-associated vasculitis was inhibited with CyA in a dose-dependent fashion (Fig. 1A).

Profile of TCLs established from MPO ANCA-associated vasculitis

Twenty one TCLs were established by cyclic stimulation with MPO antigen. All lines secreted IFN-γ which peaked on day 3 but did not secrete IL-4, similar to Con-A treated PBL. We finally focused on three lines which secreted more than 10 pg/ml of IFN-γ (Fig. 1B). These lines showed specific proliferative responses against MPO but not against β-lactoglobulin in the presence of Epstein Barr virus transformed B cell lines. Single color flow cytometry revealed that all lines highly expressed CD4 but not CD8 (Fig. 1C).

The TCL were evaluated for HLA restriction using anti-HLA mAbs; anti-DR mAb was inhibitory (> 50% inhibition), whereas anti-DQ mAb was not inhibitory, suggesting that MPO T cell responses are mainly restricted to HLA-DR (Fig. 1D).

Analysis of clonal accumulation and TCR Vβ usage in fresh PBL

PCR-SSPC analysis was used to study the T cell clonotypes with regard to the expression of particular V-D-J regions (CDR3) of the TCR-β chain, in PBL from one MPO-ANCA vasculitis patients. Each DNA sample was used to amplify individual TCR Vβ gene families and electrophoresed separately. The results indicated that there was T cell clonal accumulation and also that the accumulated T cells clones had TCR Vβ usage. In one MPO-ANCA-associated patient, we found 24 T cell clones had accumulated in fresh peripheral blood lymphocyte. The frequencies of the T cell clones bearing Vβ1, 3, 5.1, 5.2, 8, 11, 13.2, or 14 families were relatively high (Fig. 2).

Discussion

In this study, *in vitro* production of MPO-ANCA from PBL of MPO-ANCA positive patients could only be detected after stimulation with Con-A (4,5). We therefore conclude that MPO-ANCA production is dependent on stimulation of T cells. To determine whether these results were false positive or not, we performed an inhibition test. The MPO-ANCA titer of the supernatant decreased in the presence of MPO compared to that with Con-A alone, demonstrating that the antibody reactivity was specific and not a false positive.

We used phase contrast microscopy to ascertain that PBL from MPO-ANCA positive patients were viable and growing and established that the cells were alive. Thus, the inhibition of proliferation of PBL with CyA was not due to a toxic effect (6). Indirect immunofluorescence staining using culture supernatant of PBL from MPO-ANCA positive patients demonstrated a perinucle-

The T cell oligoclonalities of one typical patient with MPO-ANCA associated vasculitits.

Case O.T. MPO-ANCA: 1,000 Eunit rapidly progressive crescentic glomerulonephritis and lung bleeding by SSCP method (PBL)

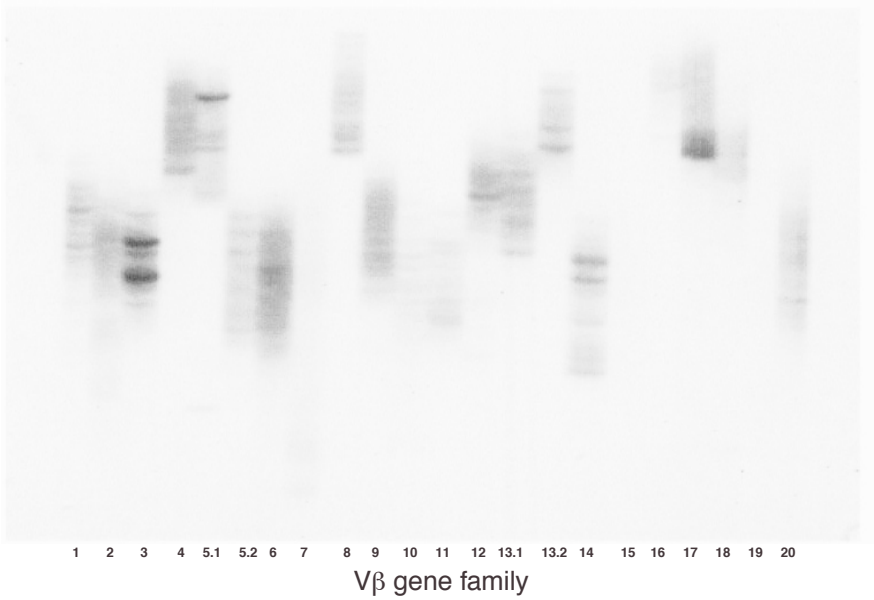

Fig. 2. Representative results of PCR-SSCP analysis of T cell clonality and TCR Vβ usages in fresh PBL of MPO-ANCA vasculitis are shown. Each DNA sample was analyzed separately for TCR Vβ gene expression. The lanes are arranged in order of the 20 Vβ gene family members (from left to right: Vβ 1-4, Vβ 5.1, Vβ 5.2, Vβ 6-12, Vβ 13.1, Vβ 13.2, and Vβ 14-20).

ar pattern on ethanol fixed neutrophils. These results suggest that T cell stimulation is required for the production of MPO-ANCA by PBL. IFN-γ was detected in the conditioned medium of PBL from MPO-ANCA positive patients, but IL-4 was not. These results suggest that Th1 cells may play an important role in MPO-ANCA associated disease etiology (7). Further additional study is needed concerning T cell stimulation and analysis of cytokine profile for the production of MPO-ANCA by PBL due to the relatively small number of our study.

However, some questions remain regarding the degree of T cell accumulation that may occur and which TCRs are expressed in these patients. For this purpose, PCR amplification using primers specific for each TCR Vβ gene could detect the usage of almost all TCR Vβ gene for autoantigen-specific T cells present in PBL (8, 9). The results of SSCP analysis argue the benefits of applying this method to the analysis of T cell clonality in patients with systemic autoimmune diseases.

The data presented here indicate that a variety of T cell clones accumulated in the lymphocyte samples taken from the MPO-ANCA vasculitis patients, suggesting that autoantigen-reactive oligoclonal T cell accumulation was present in peripheral blood from patients with MPO-ANCA-associated vasculitis.

To determine whether such T cell clones belong to Th1 or Th2 subtypes and to characterize them, we established MPO antigen-specific TCL. Characterization of TCL showed that HLA-DR-restricted Th1-type T cells may play an important role in producing MPO-ANCA in MPO-ANCA vasculitis.

Our data suggest that Th1-type T cells may play an important role in MPO-ANCA vasculitis. Recently, Popa *et al.* found that PBMC from patients with mainly proteinase-3 (PR-3) ANCA associated vasculitis produced Th2 cytokines such as IL-6 and IL-10 and only low level of IFN-γ (10). Cytokine profiles may relate to the difference between PR-3 and MPO-ANCA vasculitis. (11). Day *et al.* showed that PBMC from patients in remission produced mainly INF-γ following stimulation with PR-3, as assessed by mRNA expression and concluded a TH 1 response (12). Hellmich *et al.*, looking at PBMC in remission patients, found little INF-γ in the supernatant by ELISA (13). However, experimental conditions were probably not ideal for measuring cytokine responses, as proliferation was the prime readout.

Moreover, aiming at modulation of T cell cytokine profile in patients with ANCA associated vasculitis *in vivo* may be envisaged a possible therapeutic approach.

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