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Dynamics of Cytomegalovirus (CMV)–Specific T Cells in HIV-1–Infected Individuals Progressing to AIDS with CMV End-Organ Disease

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Background. Since cytomegalovirus (CMV) infection can cause serious clinical complications in immunocompromised individuals, we assessed cellular immune requirements for protection against CMV end-organ disease (CMV-EOD) in human immunodeficiency virus type 1 (HIV-1) infection.

Methods. Longitudinal samples from HIV-1–infected patients in the Amsterdam cohort were analyzed. Dynamics of CMV-specific CD8+ and CD4+ T cell responses were analyzed by 4-color fluorescence analysis using major histocompatibility class I CMV peptide-tetramers and by intracellular staining for perforin, granzyme B, and interferon (IFN)–γ after stimulation with CMV-specific stimuli. CMV load was measured in parallel.

Results. In individuals progressing to acquired immunodeficiency syndrome with CMV-EOD, CMV-specific IFN-γ–producing CD4+ T cells disappeared during the year before onset of CMV-EOD. This disappearance was accompanied by a sharp increase in CMV load before onset of disease. Despite increasing CMV-specific CD8+ T cell counts, decreasing CMV-specific IFN-γ–producing CD8+ T cell counts were found over time. In contrast, the percentage of CMV-specific perforin- and granzyme B–expressing CD8+ T cells increased.

Conclusions. Our data indicate that insufficient help of CD4+ T cells may cause loss of IFN-γ–producing CD8+ T cells and loss of control of CMV dissemination. Increasing CMV-infected cell counts in the face of high CMV-specific perforin- and granzyme B–expressing CD8+ T cell counts may explain the immune pathological characteristics of CMV disease.

Human cytomegalovirus (CMV) is a persistent β-herpes virus that infects >50% of the human population. After infection, the virus disseminates and remains latently present in many tissues of the host [1]. Although asymptomatic in immunocompetent hosts, in immunocompromised hosts (such as HIV-1–infected subjects), CMV infection/reinfection and reactivation can cause serious clinical complications leading to CMV end-organ disease (CMV-EOD). Cellular immunity is thought to play a crucial role in control of virus replication and prevention of disease [1]. In healthy CMV-seropositive individuals, CMV-specific CD8+ T cell counts that are heterogeneous for the capability to express interferon (IFN)–γ and perforin can be readily detected [2]. In addition, it has been shown that, over time, there is considerable variability in both CMV-specific IFN-γ–producing CD8+ T cell responses and IFN-γ–producing CD4+ T cell responses [3].

In the natural course of HIV-1 infection, CMV-EOD (as an AIDS-defining illness [4]) occurs particularly when CD4+ T cell counts decrease to <50 cells/μL. CMV-specific CD8+ T cells are present in high numbers, and a large proportion of them express tumor necrosis factor
(TNF–α and IFN-γ effector cytokines [5, 6]. In addition, CMV-specific CD4+ T cell counts are higher in HIV-1-seropositive subjects than in HIV-1-seronegative subjects, and they show a Th1-type response [7]. Higher T cell proliferative responses to CMV antigen are associated with a decreased risk of CMV retinitis [8, 9]. It has been shown in animal models and suggested in human studies that help of CD4+ T cells is associated with the activity of cytotoxic T lymphocytes (CTLs) [10]. Komanduri et al. [6] have shown an association between a decrease in CMV-specific CD4+ T cell frequencies and an inability to sustain high levels of CMV-specific CD8+ T cells in patients receiving potent combination antiretroviral therapy. In the present study, CMV-specific CD8+ T cell responses, as well as CD4+ T cell responses, were longitudinally analyzed in parallel to CMV load, in both individuals who were asymptomatic in the long term and individuals progressing to AIDS with or without CMV-EOD and without having received treatment for HIV-1 or CMV. These data provide new insights into the dynamics of CMV-specific T cells, in relation to disease progression.

SUBJECTS, MATERIALS, AND METHODS

Study population. The present study analyzed longitudinal peripheral blood mononuclear cell (PBMC) samples from participants of the Amsterdam Cohort Studies on HIV-1 infection and AIDS among homosexual men. Informed consent was obtained from all participants, and the human-experimentation guidelines of our institution were followed in the conduct of clinical research. All individuals were selected according to their HLA type (HLA-A2, which is widespread in the white population). Ten of the 19 HIV-1–seropositive individuals were designated as progressing to AIDS with CMV retinitis and/or another end-organ disease (AIDS-CMV), 4 as progressing to AIDS with opportunistic infections other than CMV (AIDS-OI), and 5 as remaining asymptomatic in the long term, with >7 years of asymptomatic follow-up and CD4+ T cell counts >400 cells/μL (classification of the Centers for Disease Control and Prevention). None of the individuals received antiretroviral or antiviral therapy during the study period. For the individuals progressing to AIDS-CMV, time points were studied from just after seroconversion to HIV-1 or study entry to the time of diagnosis of CMV. For the individuals progressing to AIDS-OI and the individuals who remained asymptomatic in the long term, the earliest time point studied was 1–2 years after seroconversion to HIV-1, and the latest time points studied were, on average, 5 years (around the time of diagnosis of AIDS) and 10 years, respectively, after seroconversion to HIV-1. Characteristics of the study participants are summarized in table 1.

HLA class I tetramer staining in parallel with intracellular IFN-γ staining, after antigen-specific stimulation. It has been well documented that the immune response to CMV in individuals expressing HLA-A2 is dominated by the NLVPMVATV (NLV) epitope of the pp65 lower matrix protein [11, 12]. HLA-A2–NLV peptide-tetrameric complexes were produced, and 4-color fluorescence analysis was performed as described elsewhere [13]. In brief, PBMCs were stained with HLA-A2–NLV tetramers. After fixation and permeabilization (Becton Dickinson), cells were stained (intracellularly) with fluorochrome-conjugated monoclonal antibodies (MAbs) against CD8, perforin (Becton Dickinson), and granzyme B (Sanquin). In parallel, PBMCs were stimulated with either 10 μg/mL NLV peptide or CMV lysate (BioWhittaker [60 μL/mL] or Microbix Biosystems [10 μL/mL]), in the presence of 2 μg/mL anti-CD28 (Sanquin) and CD49d (Becton Dickinson) MAb, for 6 h at 37°C. After 1.5 hours, 5% (wt/vol) monensin was added [13]. As a positive control for the capacity of PBMCs to produce IFN-γ, phorbol myristate acetate (PMA) and ionomycin (Sigma-Aldrich; 5 ng/mL and 1 μg/mL, respectively) were added. Unstimulated cells were used as a negative control. Cells were stored overnight at 4°C, after which time they were stained, as described above, with MAb against CD8 and IFN-γ (Becton Dickinson) or against CD4, CD3 (Becton Dickinson), and IFN-γ. Cells were fixed in Cellfix (Becton Dickinson), and up to 400,000 events were acquired by use of a FACSCalibur flow cytometer (Becton Dickinson). Lymphocytes were gated by forward-side scatter, and data were analyzed by use of CellQuest software (version 3.3; Becton Dickinson). For measurement of IFN-γ, the number of responding T cells was calculated after subtracting negative control values. Data are expressed in absolute numbers per microliter of blood, to take into account that, during HIV-1 infection and progression to AIDS, total CD4+ T cell numbers decrease dramatically.

Determination of CMV load. CMV load in PBMCs was determined by use of the Cobas Amplicor CMV Monitor kit (Roche Diagnostics), as described elsewhere [14]. In brief, PBMCs in lysis buffer were added to lysis reagent that contained a quantitation standard. DNA was precipitated with isopropanol and pelleted by centrifugation. The pellet was washed and resuspended in specimen diluent. The extracted sample was added to PCR Master Mix (Roche) and placed in the Cobas Amplicor instrument for automated amplification, detection, and quantification of CMV DNA. The limit of detection was 100 copies. Copy numbers between 1 and 100 were considered to be positive but could not be quantitated.

Statistical analysis. Wilcoxon signed rank tests were performed to compare values at early and late time points within groups. Differences between the groups were analyzed by use of the Mann-Whitney U test. Correlations were tested by use of Spearman’s nonparametric correlation test. All statistical analyses were performed by use of SPSS (version 10.0; SPSS).
<table>
<thead>
<tr>
<th>Subject</th>
<th>Classification</th>
<th>AIDS-defining illness</th>
<th>HIV-1 status</th>
<th>Months AIDS free</th>
<th>CD4$^+$ T cell count at time of diagnosis of AIDS</th>
<th>Months from AIDS to CMV</th>
<th>CD4$^+$ T cell count at time of diagnosis of CMV</th>
<th>Months from AIDS to death</th>
<th>Age at diagnosis of AIDS, years</th>
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**NOTE.** CMV, cytomegalovirus; CMV disease, CMV end-organ disease other than retinitis; LTA, individuals who remained asymptomatic in the long term; NA, not available; PCP, *Pneumocystis carinii* pneumonia; SC, seroconversion; SP, seroprevalence (these individuals seroconverted ∼0.5–1.5 years before study entry).

$^a$ HLA-B7–expressing individual.
RESULTS

**Increases of CMV-specific CD8+ T cell counts over time before onset of CMV-EOD.** HLA-A2–NLV tetramers were used to determine CMV-specific CD8+ T cell counts. Figure 1 shows the results at all time points for the individuals progressing to AIDS-CMV. To be able to analyze the data, we analyzed both early time points (defined as the earliest time point after seroconversion to HIV-1 or study entry) and late time points (on average, 10 years after seroconversion to HIV-1 in individuals who remained asymptomatic, 5 years after seroconversion to HIV-1 in individuals progressing to AIDS-OI, and 5–11 months before onset of CMV-EOD in individuals progressing to AIDS-CMV) (figure 2).

In samples from individuals who remained asymptomatic in the long term (figure 2A), HLA-A2–NLV tetramer–stained CD8+ T cells were readily detected and increased significantly during follow-up (median, 8.51–49.33 cells/μL; P = .043, Wilcoxon signed rank test). Individuals progressing to AIDS-OI (figure 2A) had relatively low and stable HLA-A2–NLV tetramer–stained CD8+ T cell counts (median, 0.73–1.39 cells/μL; P > .99, Wilcoxon signed rank test). Interestingly, in samples from individuals progressing to AIDS-CMV (figures 1A and 2A), as in samples from individuals who remained asymptomatic in the long term, HLA-A2–NLV tetramer–stained CD8+ T cells were detected at high and increasing numbers (median, 8.77–21.58 cells/μL; P = .093, Wilcoxon signed rank test). Both individuals who remained asymptomatic in the long term and individuals progressing to AIDS-CMV had higher HLA-A2–NLV tetramer–stained CD8+ T cell counts than did individuals progressing to AIDS-OI early (P = .050 and P = .024, respectively, Mann-Whitney U test) and late (P = .014 and P = .005, respectively, Mann-Whitney U test) during the course of HIV-1 infection.

**Lack of CMV-specific IFN-γ–producing CD8+ T cells in samples from individuals progressing to AIDS-CMV.** To investigate the responsiveness of CMV-specific CD8+ T cells, IFN-γ–producing CD8+ T cell counts were measured by use of intracellular IFN-γ staining, after stimulation with NLV peptide. In samples from individuals who remained asymptomatic in the long term (figure 2B), IFN-γ–producing CD8+ T cells were detected (median, 2.82–17.22 cells/μL; P = .138, Wilcoxon signed rank test), whereas, in samples from individuals progressing to AIDS-OI (figure 2B), IFN-γ–producing CD8+ T cells were present at low numbers (median, 0–0.68 cells/μL; P = .109, Wilcoxon signed rank test). In individuals progressing to AIDS-CMV (figures 1B and 2B), IFN-γ–producing CD8+ T cell counts increased toward but decreased before the first CMV-associated clinical event (median, 0–2.25 cells/μL; P = .327, Wilcoxon signed rank test). By analysis of the cumulative data on all individuals, correlations were found between HLA-A2–NLV tetramer–stained CD8+ T cell counts and IFN-γ–producing CD8+ T cell counts, both early (ρ = 0.826; P < .01, Spearman’s nonparametric correlation test) and late (ρ = 0.787; P < .01, Spearman’s nonparametric correlation test) (data not shown) during the course of HIV-1 infection.

As depicted in figure 2C, at the late time point, percentages of IFN-γ–producing cells within HLA-A2–NLV tetramer–stained CD8+ T cells were lowest in samples from individuals progressing to AIDS-CMV (median, 6%)—the median percentage in samples from individuals who remained asymptomatic in the long term was 28% and that in samples from individuals progressing to AIDS-OI was 58%—and differed at the late time point between individuals who remained asymptomatic in the long term and individuals progressing to AIDS-CMV (P = .019, Mann-Whitney U test). Thus, despite high CMV-specific HLA-A2–NLV tetramer–stained CD8+ T cell counts before onset of CMV-EOD, a relatively low proportion of these cells was able to produce IFN-γ after stimulation with NLV peptide.

**High percentages of CMV-specific perforin- and granzyme B–expressing CD8+ T cells in individuals progressing to AIDS-CMV.** To investigate the potential cytotoxic capacity of CMV-specific HLA-A2–NLV tetramer–stained CD8+ T cells, expression of perforin and granzyme B within these cells was analyzed. In samples from all groups, percentages of single perforin-expressing T cells were low, whereas single granzyme B–expressing T cells were detected abundantly in the CMV-specific CD8+ T cells (data not shown). In addition, T cells not expressing perforin and granzyme B were detected in samples from all groups. In samples from individuals who remained asymptomatic in the long term (figure 2D), high percentages of perforin- and granzyme B–expressing T cells were detected (median, 24.40%–43.75%; P = .138, Wilcoxon signed rank test). In samples from individuals progressing to AIDS-OI (figure 2D), these percentages were lower and were stable (median, 19.45%–19.89%; P > .99, Wilcoxon signed rank test). In samples from individuals progressing to AIDS-CMV (figures 1C and 2D), the percentage of these cells started at percentages similar to those in samples from individuals progressing to AIDS-OI and increased toward the time of onset of CMV-EOD (median, 22.75%–43.75%; P = .080, Wilcoxon signed rank test). For all individuals, HLA-A2–NLV tetramer–stained CD8+ T cell counts and perforin- and granzyme B–expressing HLA-A2–NLV tetramer–stained T cell counts correlated significantly (early, ρ = 0.990 and P < .01, Spearman’s nonparametric correlation test; late, ρ = 0.884 and P < .01) (data not shown). Interestingly, at the late time point, samples from individuals progressing to AIDS-OI had significantly lower percentages of CMV-specific perforin- and granzyme B–expressing CD8+ T cells, compared with those from individuals progressing to AIDS-CMV (P = .050, Mann-Whitney U test). Thus, in individuals progressing to CMV-EOD, even though a relatively low percentage of CMV-specific
Figure 1. Dynamics of cytomegalovirus (CMV)–specific CD8+ T cells, CD4+ T cells, and CMV load during the course of HIV-1 infection in individuals progressing to AIDS with CMV retinitis and/or another end-organ disease (AIDS-CMV). T cell responses were analyzed in samples from individuals progressing to AIDS-CMV. Samples from patients were analyzed for nos. of HLA-A2–NLV tetramer–stained CD8+ T cells per microliter of blood (A), nos. of interferon (IFN)–γ–producing CD8+ T cells per microliter of blood after stimulation with NLV peptide (B), percentage of perforin- and granzyme B–expressing cells within HLA-A2–NLV tetramer–stained CD8+ T cells (C), nos. of IFN-γ–producing CD4+ T cells per microliter of blood after stimulation with CMV lysate (D), and CMV load, in copies per 10^6 peripheral blood mononuclear cells (the dotted line represents the limit of detection) (E). Because of the variation with respect to the time to CMV end-organ disease (CMV-EOD) (e.g., light blue line vs. green line in A), the data are plotted retrospectively from the time of onset of CMV-EOD. Each color represents the same patient in different analyses.

CD8+ T cells was capable of producing IFN-γ, expression of perforin and granzyme B was not impaired.

Loss of CMV-specific IFN-γ–producing CD4+ T cells during the year before the first CMV-associated clinical event. CMV-specific CD4+ T cells were studied by intracellular expression of IFN-γ after stimulation with CMV lysate. In samples from individuals who remained asymptomatic in the long term (figure 2E), IFN-γ–producing CD4+ T cells were readily detected (range, 0–75.58 cells/μL) and were stable (median, 0.88–0.11 cells/μL; P = .893, Wilcoxon signed rank test). In samples
Figure 2. Comparison of early and late time points of cytomegalovirus (CMV)–specific T cell responses between groups. Early and late time points from the longitudinal data on individuals who remained asymptomatic in the long term (LTA), individuals progressing to AIDS with opportunistic infections other than CMV (AIDS-OI), and individuals progressing to AIDS with CMV retinitis and/or another end-organ disease (AIDS-CMV) were compared by use of the Wilcoxon signed rank test; 2-tailed P values are depicted in the upper portion of the panels. Depicted are nos. of HLA-A2–NLV tetramer–stained CD8+ T cells per microliter of blood (A), nos. of interferon (IFN)–g–producing CD8+ T cells per microliter of blood after stimulation with NLV peptide (B), percentage of IFN–g–producing cells within HLA-A2–NLV tetramer–stained CD8+ T cells (C), percentage of perforin- and granzyme B–expressing cells within HLA-A2–NLV tetramer–stained CD8+ T cells (D), and nos. of IFN–g–producing CD4+ T cells per microliter of blood after stimulation with CMV lysate (E). Dots represent individual patients, and the median is shown as a bar.

from individuals progressing to AIDS-OI (figure 2E), IFN–g–producing CD4+ T cells were detected, although at lower levels (median, 0.08–0.51 cells/μL; P = .285, Wilcoxon signed rank test). Remarkably, in samples from individuals progressing to AIDS-CMV (figures 1D and 2E), even though IFN–g–producing CD4+ T cell counts were detected early during infection (range, 0–2.71 cells/μL), they decreased significantly over time, from a median of 0.30 to 0 cells/μL (P = .028, Wilcoxon signed rank test). However, no correlation could be found between IFN–g–producing CD8+ T cell counts and IFN–g–producing CD4+ T cell counts in the data on all HIV-1–seropositive individuals combined. Also, absolute numbers of CD4+ T cells and IFN–g–producing CD8+ T cells did not correlate.

The disappearance of IFN–g–producing CD4+ T cells in individuals progressing to AIDS-CMV could not be explained by an absolute lack of responsiveness of CD4+ T cells, since stimulation with PMA and ionomycin readily induced expression of IFN–g by CD4+ T cells, both in terms of absolute numbers and percentages (data not shown). Thus, the disappearance of CMV-specific IFN–g–producing CD4+ T cells in individuals progressing to AIDS-CMV was associated specifically with the development of CMV disease.
Detectable CMV load a few months before onset of CMV-EOD. To investigate the role of CMV replication, CMV load was measured in a large number of PBMC samples. No CMV load was detectable in samples from individuals who remained asymptomatic in the long term, at the later time point, CMV load was detectable in a few samples from individuals progressing to AIDS-OI but at levels too low to be quantified (data not shown). CMV load was detectable in samples from all individuals progressing to AIDS-CMV. In samples obtained from 8 of 10 individuals during the year before onset of CMV-EOD, CMV load could be quantified (range, 148–18,184 copies/10⁶ PBMCs; median, 439 copies/10⁶ PBMCs; figure 1E). Although CMV load did not correlate with the CMV-specific CD8 or CD4 response, it did correlate with disease progression, since CMV load was detected in quantifiable amounts only in samples from individuals progressing to AIDS-CMV.

DISCUSSION

In the present study, to investigate which factors might contribute to CMV-associated disease progression, longitudinal samples from individuals who remained asymptomatic in the long term, individuals progressing to AIDS-OI, and individuals progressing to AIDS-CMV were analyzed in terms of CMV-specific CD8⁰ and CD4⁺ T cell responses in parallel to CMV load in PBMCs. Staining with HLA-A2–NLV tetramer clearly showed that CMV-specific HLA-A2–NLV tetramer–stained CD8⁺ T cells were present in samples from all individuals and increased over time both in samples from individuals who remained asymptomatic in the long term and in samples from individuals progressing to AIDS-CMV. These increasing numbers of CMV-specific T cells might have been induced by episodes of CMV reactivation. CMV load was indeed detectable and much increased in samples from the individuals progressing to AIDS-CMV before diagnosis of CMV. This may suggest that individuals who remain asymptomatic in the long term are successful at keeping CMV load below the limit of detection during follow-up, whereas individuals progressing to AIDS-CMV fail to control CMV replication. Individuals progressing to AIDS-OI were shown to have low CMV-specific T cell counts, which fits the low virus-specific T cell counts in general [15, 16]. This may be explained by a lower capacity to proliferate in response to viral antigens, as has been shown for HIV-1–specific CD8⁺ T cells [17]. That very low levels of CMV DNA were detectable at some of the later time points and that no clinical CMV-related symptoms were observed suggest that CMV reactivation may simply be less severe, despite the low CMV-specific CD8⁺ T cell counts.

We used IFN-γ as a functional readout and showed much lower percentages of IFN-γ-producing CD8⁺ T cells within HLA-A2–NLV tetramer–stained CD8⁺ T cells in samples from individuals progressing to AIDS-CMV than in samples from either individuals progressing to AIDS-OI or individuals who remained asymptomatic in the long term, suggesting CMV-specific CD8⁺ T cell dysfunction. Indeed, for 3 HIV-1–infected individuals with low CD4⁺ T cell counts or no CD4⁺ T cells at all, dysfunction of HIV-1- and CMV-specific CD8⁺ T cells was shown [18]. Likewise, this has been described for HIV-1–specific CD8⁺ T cells in individuals progressing to AIDS [19] and for Epstein-Barr virus–specific CD8⁺ T cells in HIV-1–infected individuals progressing to AIDS-related non-Hodgkin lymphoma [15]. In contrast, the percentages of CMV-specific perforin- and granzyme B–expressing CD8⁺ T cells were highest in samples from individuals progressing to AIDS-CMV, indicating that these individuals did have a substantial number of CTLs with cytotoxic capacity [5].

Low CMV-specific IFN-γ-producing CD8⁺ T cell counts, in combination with high perforin- and granzyme B–expressing CD8⁺ T cell counts, indicate that, in CMV-specific T cells, release of IFN-γ and expression of perforin and granzyme B are not necessarily linked [20]. Since CMV is a typical cytopathic virus [1] that has been shown to depend more on the antiviral effect of cytokines, such as IFN-γ and TNF-α, than on the perforin-dependent granule exocytosis pathway [21], the decreased capacity of CMV-specific cells to produce IFN-γ may be crucial.

The most conspicuous finding in the present study was that, in the individuals progressing to AIDS-CMV, CMV-specific IFN-γ–producing CD8⁺ T cells disappeared during the year before onset of CMV-EOD, suggesting that CMV-specific IFN-γ–producing CD8⁺ T cells could play an important role in protection against CMV-EOD. Dysfunction of CTLs (in terms of IFN-γ) and memory B cells has been shown to be associated with simian immunodeficiency virus–induced impairment of CMV-specific CD4⁺ T helper cells in rhesus macaques [22], and CMV-specific IFN-γ has been shown to act as an immunological predictor of CMV control [23]. However, no correlation was found between IFN-γ–producing CD8⁺ T cell and CD4⁺ T cell counts, as has been shown in healthy donors but not in asymptomatic CMV-seropositive renal transplant recipients receiving basic immunosuppressive drug therapy [24]. Possibly, the IFN-γ produced by the CMV-specific CD4⁺ T cells may play a role in controlling CMV directly, as does IFN-γ produced by CD8⁺ T cells.

In conclusion, our data show that individuals progressing to AIDS-CMV lose CMV-specific IFN-γ–producing CD4⁺ T cells during the year before onset of CMV-EOD in parallel with a sharp increase in CMV load. CMV-specific CD8⁺ T cells remain in high numbers, with a high proportion of these cells expressing perforin and granzyme B and a low proportion producing IFN-γ. The decrease in production of IFN-γ in the CD8 compartment may be due to insufficient numbers of CD4 Th1 cells and may allow for CMV dissemination. High viral load leads to high CMV-infected cell counts and induces high per-
forin- and granzyme B–expressing HLA-A2–NLV tetramer–stained CD8$^+$ T cell counts. These high cytotoxic T cell counts, in combination with the high infected-cell counts, may lead ultimately to disease in the infected organ(s), irrespective of the type of CMV-EOD (data not shown). Our data suggest that the balance between CD4$^+$ T cells, CD8$^+$ T cells, IFN-γ, and perforin and granzyme B is very important to the control of CMV replication and the prevention of virus-associated immune pathological characteristics.

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**References**