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# Monitoring of Leukocyte Cytomegalovirus DNA in Bone Marrow Transplant Recipients by Nested PCR

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**A nested PCR assay for the detection of human cytomegalovirus (CMV) DNA was evaluated by weekly monitoring of blood samples taken from 101 bone marrow transplant (BMT) recipients. When peripheral blood leukocytes were used as the source of CMV DNA, even a modified assay with stringent temperature-cycling conditions was as sensitive as the standard assay. The sensitivity, specificity, and positive predictive value of two consecutively positive leukocytic PCR results with this modified assay in predicting CMV disease of 101 patients submitting 1,441 peripheral blood leukocyte samples were found to be 92.1, 63.5, and 60.3%, respectively. The positive predictive value of patients' seropositivity for CMV was 40%, while that of viremia was 72%. However, viremia followed CMV disease by a median of 1.5 days, while the first leukocytic positive PCR assay preceded disease by a median of 14 days. By use of the criteria of two consecutively positive PCR results instead of recipient CMV seropositivity for starting preemptive ganciclovir treatment, 38 of the 43 recipients with isolated single positive or negative assays (groups I and II) would be spared unnecessary ganciclovir treatment. Moreover, two other findings support the use of antiviral prophylaxis before engraftment in high-risk cases and subsequent preemptive treatment of patients with two consecutively positive PCR assays. First, for 7.9% of 76 patients with positive assays (groups II and III), the first positive PCR assay occurred before engraftment, which implied the presence of viral DNA in the blood (DNAemia) soon after transplantation. Second, isolated single positive assays which were clustered around the second to sixth weeks after transplantation were found for 18 patients (group II) and could represent abortive episodes of CMV infection.**

Human cytomegalovirus (CMV) is known to cause mild or asymptomatic infection in most healthy individuals but can cause symptomatic disease in congenitally infected or premature neonates and in some immunosuppressed hosts. Increasing acceptance and use of bone marrow transplantation (BMT) as a form of curative treatment for patients with hemic malignancies are associated with a substantial increase in CMV diseases during the first 3 months after the transplant. The availability of antiviral agents and the high fatality of CMV pneumonitis despite therapy have stimulated the search for markers identifying patients with progressing CMV infections (16). CMV seropositivity of donors' marrow or blood products and being a seropositive BMT recipient are known to be independent risk factors for CMV disease (11). Shedding of CMV in urine or saliva was shown to be a risk factor but with poor predictive value. However, the presence of CMV in peripheral blood leukocytes (PBL) or bronchoalveolar lavage fluid is an important predictor of CMV diseases (12, 18). Until recently, the detection of CMV in these clinical specimens depended on the detection of early antigen expression in shell vial cultures or isolation in conventional cell cultures, which may take 2 and 14 days, respectively. The availability of more rapid methods of detection of CMV DNA or its antigen in buffy coat and/or plasma (20, 21) would allow early institution of preemptive therapy. Such a strategy could avoid the cost and side effects of routine antiviral prophylaxis in all seropositive patients with or without urinary or salivary shedding of CMV (1, 2). A longitudinal study of the clinical use and the prognostic significance of a nested PCR assay for the detection of leukocytic CMV DNA is reported. The clinical characteristics, viremic load, and other risk factors affecting the occurrence of CMV disease and their timing were analyzed in detail to provide a more rational basis for the design of preemptive antiviral therapeutic strategy.

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## MATERIALS AND METHODS

**Patients.** A total of 101 patients admitted for BMT between April 1991 and December 1993 were included in this study. The CMV serological statuses of the donors and recipients were determined by a serum enzyme-linked immunosorbent assay (Abbott Laboratories) before transplantation. The clinical and serological statuses of the patients are shown in Table 1.

**Specimen collection and CMV culture.** Ten milliliters of heparinized blood was collected from each patient at weekly intervals for the first 120 days after transplantation. PBL were obtained by mixing the heparinized blood with an equal volume of 6% dextran suspended in phosphate-buffered saline (PBS). After sedimentation at 37°C for 30 min, the upper layer was removed and centrifuged at 900 × *g* for 10 min. The residual erythrocytes in the pellet were then lysed with hypotonic saline twice at 4°C for 3 min. After a washing with PBS, PBL were resuspended at $2 \times 10^6$ cells per ml in maintenance medium (Eagle's minimal essential medium supplemented with 2% fetal calf serum) for CMV culture or in water for CMV PCR assay. Liver, gastric, duodenal, and colonic biopsy, bronchoalveolar lavage fluid, and cerebrospinal fluid samples were obtained from patients with suspected CMV diseases. For every specimen, tube cultures of human embryonic lung fibroblasts were inoculated and grown in maintenance medium. The cultures were monitored for 6 weeks for the presence of a specific CMV cytopathic effect which was confirmed by immunofluorescence.

**DNA extraction and PCR.** After being boiled for 15 min, 50 μl of the PBL suspension was used for PCR. A nested PCR which was essentially a modification of a procedure described previously (8) was carried out. For amplification, two sets of primers were used to generate a 168-bp fragment (external primers CMTR1 [5′-CTG TCG GTG ATG GTC TCT TC-3′] and CMTR2 [5′-CCC GAC ACG CGG AAA AGA AA-3′] and internal primers CMTR3 [5′-TCT

TABLE 1. Clinical characteristics of BMT recipients and their disease correlations with leukocyte PCR or CMV viremia

| Characteristic | Value for group: | | |
|---|---|---|---|
| | I (no positive PCR) | II (isolated single positive PCR) | III (consecutively positive PCRs) |
| Total no. of patients | 25 | 18 | 58 |
| Sex (female/male) | 8/17 | 10/8 | 28/30 |
| Median age (range), yr | 28 (14–44) | 32 (17–48) | 32 (17–50) |
| Diagnosis of[a]: | | | |
| AML | 6 | 8 | 21 |
| CML | 8 | 4 | 18 |
| Other | 11 | 6 | 19 |
| CMV status of donors/recipients | | | |
| Positive[b]/Positive | 12 | 13 | 52 |
| Negative[c]/Positive | 9 | 4 | 5 |
| Positive/Negative | 2 | 0 | 1 |
| Negative[c]/Negative | 2 | 1 | 0 |
| BMT type: | | | |
| Allogeneic[b] | 15 | 14 | 51 |
| Autologous[b] | 7 | 4 | 1 |
| Matched unrelated | 2 | 0 | 6 |
| Syngeneic | 1 | 0 | 0 |
| No. of patients receiving total-body irradiation | 11 | 12 | 40 |
| Acute GVHD[b] (grade II to IV) | 8 | 2 | 36 |
| CMV syndromes[b]: | | | |
| Total no. of episodes[d] | 0 | 3 | 46 |
| Leukopenia or thrombocytopenia | 0 | 3 | 17 |
| Pneumonitis | 0 | 0 | 13 |
| Hepatitis | 0 | 0 | 9 |
| Gastroenterocolitis | 0 | 0 | 5 |
| Retinitis | 0 | 0 | 1 |
| Encephalitis | 0 | 0 | 1 |
| Blood samples monitored | 380 | 282 | 779 |
| Positive PCR assays[b] (%) | 0 | 22 (7.8) | 275 (35.3) |
| Viremic patients[b] (CMV isolates) | 0 | 0 | 25 (49) |
| No. of patients with CMV disease[b] (%) | 0 | 3 (16.7) | 35 (60.3) |

[a] AML, acute myeloid leukemia; CML, chronic myeloid leukemia.
[b] Significant differences between group I or II and group III ($P < 0.05$) were shown by chi-square test.
[c] Including autologous BMT.
[d] More than one episode and syndrome can occur in a single patient.

CTG GTC CTG ATC GTC TT-3′] and CMTR4 [5′-GTG ACC TAC CAA CGT AGG TT-3′]). They were located within morphological transforming region II (15). The oligonucleotide MTR, 5′-TGA CGC GGA GAT CTT GCA ATC TGG TCG CGT-3′, was used as a probe for confirmation after Southern blotting for the first 100 specimens. In all PCR assays, 100 copies of a cloned amplicon produced by the CMV external primers were routinely used as a positive control and water was used as a negative control. Procedures for preventing false positives were strictly followed (9). To control for false-negative results due to the presence of inhibitors from each sample, a β-globin control was also included in parallel with samples (17).

**The standard PCR assay.** Immediately before PCR, DNA extracts and controls were denatured at 95°C for 15 min and then chilled on ice for 5 min. For the initial amplification by the external primers, 50 μl of the denatured leukocyte DNA was amplified in a 100-μl reaction mixture containing sample DNA, PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.01% gelatin, 3 mM $MgCl_2$ [pH 8.3]), 200 μM deoxynucleoside triphosphates, 0.5 μM each external primer, and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Each sample was amplified in 30 cycles of 94, 55, and 72°C for 1 min each and extended for 10 min at 72°C after the last cycle in an automated thermal cycler (Perkin-Elmer Cetus, Gouda, The Netherlands). For nested amplification by the internal primers, 10 μl of the first PCR product was transferred to a new reaction mixture containing PCR buffer, 200 μM deoxynucleoside triphosphates, 0.5 μM each internal primer, and 2.5 U of *Taq* polymerase. Amplification was continued for 30 cycles of 94, 55, and 72°C for 1 min each and extended for 10 min at 72°C after the last cycle. By electrophoresis, 10 μl of the final PCR product was analyzed on a 2% agarose gel (Promega, Madison, Wis.) in 1× Tris-borate-EDTA buffer. A molecular size marker (ϕX174 DNA *Hae*III digest; Promega) was also run on each gel. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg/ml) for 15 min, washed, and photographed under UV illumination. The sensitivity of this protocol was determined by serial dilution of the cloned amplicons produced by the external primers. The sensitivity limit was estimated to be five copies.

**The modified PCR assay.** During the initial comparative study of standard and modified leukocyte PCR assays of 210 specimens from the first 20 patients, a modified PCR assay condition was evaluated concurrently. During the initial amplification by the external primers, 100 μM deoxynucleoside triphosphates, 0.05 μM each external primer, and 0.375 U of *Taq* polymerase were used instead. The number of cycles was decreased to 20. The conditions used in the subsequent amplification by internal primers were unchanged. The sensitivity limit was determined to be 10 to 100 copies. Only this PCR assay was used for detection of leukocyte CMV DNA in the subsequent study.

**Clinical correlation.** Criteria for diagnosis of CMV disease were used as described previously (14). Briefly, CMV pneumonia was defined by compatible radiographic findings and evidence of CMV in bronchoalveolar lavage fluid as demonstrated by conventional culture or DEAFF (detection of early antigen fluorescent foci) test. CMV gastroenterocolitis was defined by clinical symptoms, endoscopic evidence of inflammation, and detection of CMV in the biopsy specimens. CMV retinitis was diagnosed by the typical fundoscopic finding, whereas encephalitis was diagnosed by positive CMV cultures of cerebrospinal fluid samples from patients with unexplained encephalopathy. CMV hepatitis was defined as CMV viremia and unexplained elevation of hepatic parenchymal enzyme levels with or without confirmation by liver biopsy. When autopsies were performed, the finding of typical owl eye inclusions with positive CMV antigen by immunocytochemical staining was considered evidence of CMV disease. Patients with these symptomatic diseases were given intravenous ganciclovir (5 mg/kg of body weight twice daily) for 14 days and then given maintenance treatment while on immunosuppression for graft-versus-host disease (GVHD).

## RESULTS

We first evaluated 210 blood samples from 20 patients to compare leukocyte PCR assays under different temperature-cycling conditions. When the leukocyte CMV culture was used

TABLE 2. Comparison of conventional CMV culture and PCR assays under different amplification conditions

| Leukocyte CMV culture result | *n* | No. of positive leukocyte PCR results (%)[a] | |
|---|---|---|---|
| | | Standard assay | Modified assay |
| Positive | 15 | 15 (100) | 15 (100) |
| Negative | 195 | 62 (31.8) | 59 (30.3) |

[a] No statistically significant difference between the two assays.

as the "gold standard," the leukocyte PCR was found to detect all viremic specimens when either the more sensitive standard assay or the more stringent modified assay was used (Table 2). A high background was observed when the leukocyte PCR was performed under the standard conditions (Fig. 1); therefore, leukocyte PCR assays under the modified conditions were used in the rest of the study.

For the 101 patients serially monitored, the results of the CMV PCR assays, viremia, and occurrence of disease were correlated with each other. The times of peak incidence of CMV DNAemia and viremia were also compared. The results are shown in Fig. 2. When PCR results were analyzed with regard to CMV disease, 25 patients (24.8%) never had a positive PCR assay (group I) and none developed CMV disease. Among the 18 patients (17.8%) who had only an isolated positive PCR result (group II) during the first 120 days posttransplant, only three episodes of CMV disease occurred. All three episodes were self-limiting and manifested as a sudden drop in the leukocyte and platelet counts only. The remaining 58 patients (57.4%; group III) had positive PCR assays in 2 to 13 consecutive weekly samples (median = 4). Within this group, 35 patients eventually developed CMV disease and had a median number of positive PCR assays of 5 (range, 3 to 13), whereas 23 patients remained disease-free and had a median number of positive PCR assays of 3 (range, 2 to 10).

**Correlation of PCR positivity with viremia.** Virus isolation was negative for all specimens from patients in groups I and II. Within group III, the numbers of viruses isolated from blood from the 35 patients with disease and 23 patients without disease were 36 and 13, respectively (one patient might have 1 to 8 isolates). Altogether, 25 patients (43.1%) in group III exhibited viremia.

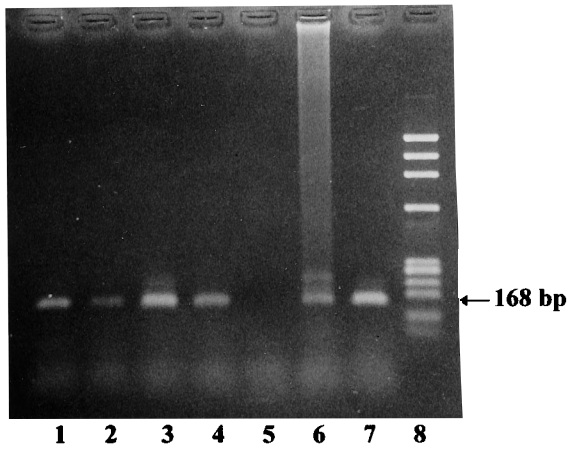


FIG. 1. Typical findings of the nested PCR assay for CMV DNA upon gel electrophoresis. Lanes 1 and 3, 10 and 100 copies, respectively, of CMV genome detected by standard leukocyte PCR assays; lanes 2 and 4, 10 and 100 copies, respectively, of CMV genome detected by modified leukocyte PCR assays; lane 5, negative control; lanes 6 and 7, CMV DNA detected in a blood specimen by standard and modified leukocytic PCR assays, respectively; lane 8, molecular weight marker (ϕX174 DNA *Hae*III digest).

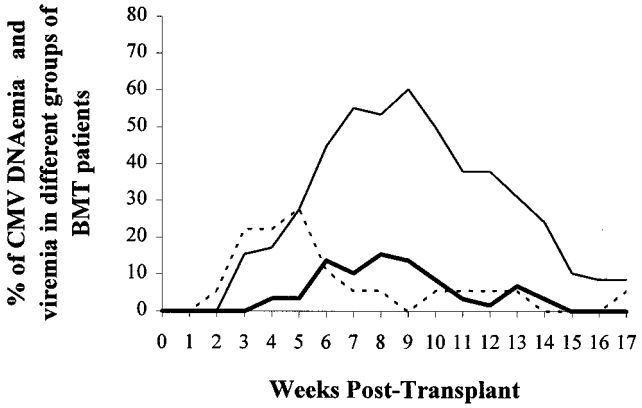


FIG. 2. Time course of CMV viremia and CMV DNAemia as manifested by positivity in the modified leukocyte PCR assay. Results for CMV viremia in group III patients (▬) ($n$ = 58) and CMV DNAemia in group II (– – –) ($n$ = 18) and group III (——) ($n$ = 58) patients are shown.

**Temporal relationship of CMV disease, PCR positivity, and viremia.** Most patients developed CMV disease within the first 100 days (Fig. 3), with the onset of disease preceded by PCR positivity by 0 to 61 days (median = 14 days). Unexpectedly, 6 of 76 patients (groups II and III) had their first positive PCR result before engraftment. The incidence of PCR positivity peaked at the fifth week posttransplant for group II and at the ninth week for group III (Fig. 2). As for viremia, there were 30 episodes which occurred exclusively in group III patients. The incidence of viremia peaked at the eighth week, a median of 10 days (range, 0 to 49 days) after the incidence of PCR positivity. However, viremia tended to occur at a median of 1.5 days after the occurrence of CMV disease, since not all viremic patients had CMV disease and vice versa.

**Clinical parameters and development of CMV disease (Table 1).** No significant difference could be shown with respect to age, sex, and diagnosis, but significantly more patients in group III than in groups I and II had allogeneic transplants, grade II to IV GVHD, and a positive CMV serological status for both the donor and the recipient. Overall, the presence of two or more consecutively positive PCR assays had a 92.1% sensitivity of predicting CMV disease and a specificity of 63.5%. The positive predictive value (PPV) of CMV disease by this diagnostic criterion was 60.3%. This compared favorably with recipients' seropositivity for CMV, which had a PPV of 40%. The PPV of viremia was 72%, but viremia followed CMV disease by a median of 1.5 days and thus had no value for the decision whether to use preemptive therapy. Table 3 shows different patient categories according to viremic load. Those with persistent PCR positivity and positive cultures of CMV in blood would have high viremic loads and were associated with greater relative risk for CMV disease.

## DISCUSSION

The availability of a rapid and noninvasive test for predicting CMV disease in BMT recipients is essential for targeting patients who would benefit from preemptive antiviral therapy while sparing others the cost and toxicity of the drug treatment. Obviously, multiple blood samples are preferable to a single fluid sample taken during a bronchoalveolar lavage procedure, which is uncomfortable, invasive, and demands the availability

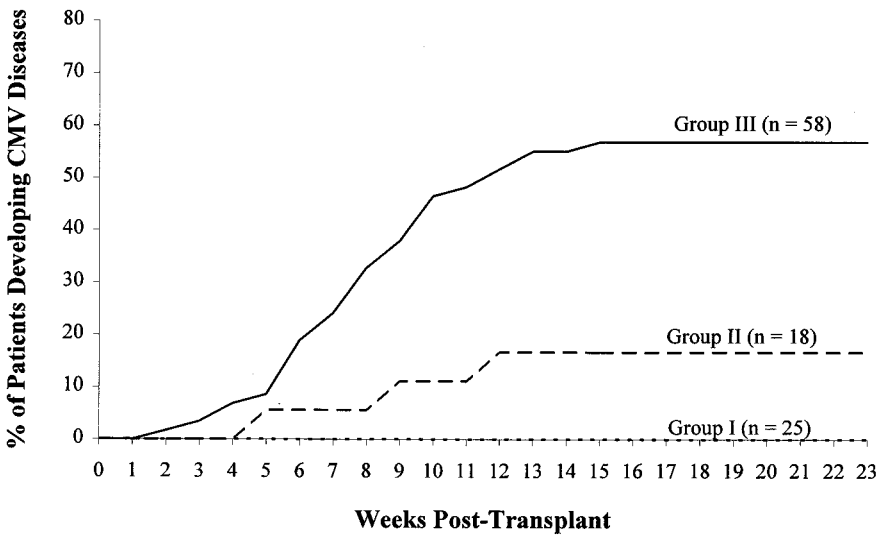


FIG. 3. Cumulative frequency of CMV disease in the three groups of BMT patients within the first 160 days posttransplant.

of well-trained bronchoscopists. Using PCR techniques on blood samples, nine studies involving 4 to 83 BMT recipients have been reported. All of them utilized a single-step PCR assay followed by probing, but various blood components were used, including PBL (3–7, 21), plasma (10, 22), and serum (19). In the present study, a nested PCR assay that circumvented the need for DNA probing was used for weekly monitoring of 101 BMT patients. However, the presence of smearing and multiple bands in 30% of the leukocyte PCR assays under standard conditions defeats the purpose of the nested PCR, which should eliminate the need for confirmation by probing. This problem was solved by using the modified assay with reduction of external primers, deoxynucleoside triphosphate, and *Taq* polymerase during the first 20 cycles. Similar sensitivity was achieved while the presence of nonspecific amplifications was eliminated. Thus, only the modified PCR assay utilizing PBL DNA extract was used in the remaining part of this study.

The time course of CMV DNAemia within the first 120 days after BMT reported in this study would provide a rational basis for the design of preemptive antiviral therapeutic strategy. In this comprehensive study of 101 consecutive patients undergoing BMT, three different subsets of patients could be delineated. When the percentage of PCR positivity was correlated with time, the peak occurred within days 30 to 120, which coincided well with the timing of CMV viremia and disease (Fig. 2 and 3). The single positive results (group II) could not be false positive, since they clustered around the second to sixth weeks (Fig. 2). These isolated single positive results may represent abortive CMV disease episodes which were effectively contained by the host defense mechanism at the early stage of infection. Even within group III, up to 39% of patients did not develop CMV disease. This is in keeping with previous reports of positive PCR assays and/or viremia but an absence of disease in patients who had no remarkable decrease in $CD4^+$ T cells (6, 10). Early DNAemia before marrow engraftment was found in six patients. This finding, together with the clustering of isolated single positive results between the second and sixth weeks, may imply active viral replication and dissemination early in the course of the transplant due to the immunosuppression after conditioning by total-body irradiation and high-dose chemotherapy. Such findings are compatible with a previous report that intravenous acyclovir within the first 30 days significantly decreased the incidence of CMV infection and disease between days 30 and 120 (13). The early DNAemia may persist or abort, as determined by the host $CD4^+$ T-cell count, which is affected by the use of anti-GVHD immunosuppression after engraftment. This hypothesis is supported by the higher incidence of grade II to IV GVHD (Table 1) in group III than in group II (62 versus 11%).

TABLE 3. Correlation of risk of CMV disease with viremic load as expressed by the number of positive leukocyte PCR assays and CMV isolation

| Patient group[a] | No. of positive PCR assays | CMV isolation | No. of patients | | Relative risk odds ratio (95% CI)[b] |
|---|---|---|---|---|---|
| | | | Total | With CMV disease | |
| I | 0 | − | 25 | 0 | 0 |
| II | 1 | − | 18 | 3 | 0.27 (0.06–1.12) |
| III | ≥2 | − | 33 | 17 | 2.4 (0.9–6.1) |
| "III" | ≥2 | + | 25 | 18 | 7.2 (2.4–22.6) |

[a] Statistically significant differences in incidence of CMV disease between patient groups III and I or II ($P < 0.05$) but not between groups III and "III" were determined by chi-square test with Yates' correction.
[b] 95% CI, 95% confidence interval.

The viremic load as reflected by different combinations of virological findings was found to be highly correlative with the risk of CMV disease (Table 3). Patients with consecutively positive PCR assays and viral isolation (group "III") were likely to be those in whom the viral burden in the blood was the heaviest and most persistent, whereas those who had an isolated single positive PCR assay but not a positive viral isolation (group II) probably had the lowest degree and duration of viremia and thus a low risk of CMV disease. In addition, since no significant difference in occurrence of CMV disease between patient groups III and "III" (Table 3), both with two consecutively positive PCR assays, could be shown, this criterion may be used as the sole marker to guide preemptive therapy.

Of the 12 autologous transplant patients included in this study, only 1 who did not develop any disease had consecutively positive PCR assays. One patient was seronegative and had an isolated single positive PCR assay. This was probably

related to the failure of the blood product transfusion filters (PALL Biomedical Ltd.) which were used to prevent CMV infection from unscreened blood products. None of the patients had symptomatic diseases. Again, this is in keeping with others' experience that the incidence of CMV disease is lower in autologous transplant recipients. The availability of this assay is important in Hong Kong, since most of our BMT recipients (94.1%) are seropositive for CMV. Up to 38 patients in this study would have been spared ganciclovir treatment if the criterion of consecutively positive PCR assays rather than seropositivity had been used for deciding on preemptive therapy.

In conclusion, the nested PCR assay could be used to replace a single-step PCR followed by probing. The presence of two consecutively positive leukocyte PCR results in weekly assays within the first 120 days post-BMT is a sensitive indicator for anti-CMV prophylaxis, allowing in addition an ample time margin (median of 7 days before disease onset) for drug action. Further evaluation of this strategy for preemptive treatment should be undertaken.

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