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Immune reactive cells in senile plaques and cognitive decline in Alzheimer’s disease

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Abstract

We examined the associations of postmortem neocortical immunoreactivities for microglia, astrocytes, Aβ-peptide, and Tau with cognitive changes in clinically characterized subjects with pathological diagnoses (CERAD classification) of definite AD (9), possible AD (15) and age-matched controls (11). By measuring the fractional area (FA) of immunoreactivity, we found that Aβ deposits appear early in the pathogenesis of Aβ, but cannot account for cognitive decline. We found a significant increase in FA for microglia in possible AD cases (nondemented) compared to controls (P<0.05) and in FA for astrocytes in definite AD (demented) compared to possible AD (P<0.01). Tau immunoreactivity was observed only in the neuropil of definite AD cases (P<0.001). The significant increase in microglia between controls and AD possible cases suggests that activation of microglia occurs in the early pathogenesis of AD, whereas the significant association between astrocytic reaction and dementia, suggests that these cells play a role in the late stage of the disease, when dementia develops. Tau immunoreactivity appears as the strongest morphological correlate of dementia.

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Keywords: Astrocite; Aβ-peptide; Dementia; Immunity; Microglia; Tau-protein

1. Introduction

In Alzheimer’s disease (AD), amyloid β-peptide (Aβ-peptide) is deposited in the brain parenchyma and cerebral blood vessels. Senile plaques (SP) composed of Aβ, neurofibrillary tangles (NFTs), and neuritic and synaptic abnormalities are the central pathological features of AD. Although the neuropathology of end stage AD is well known [2,11,25,30,34,51], the earlier stages of the disease are not as clearly defined and need further study. Because SPs are present in some individuals with well-documented normal cognition [7,9,22,43], it is important to ask whether the constituents of SPs and the reaction to these lesions in nondemented and demented subjects are the same. There is growing evidence for a chronic immune response in the brains of AD patients and that this response may contribute significantly to the damage and degeneration of neurons and neurites leading to dementia [31,32].

For example, SPs contain microglia and astrocytes, both of which are immunocompetent cells [8,10,34,45], as well as complement proteins [1,31,46]. Microglia are the principal immune cells in the CNS, and have the ability to phagocytize and to produce superoxide, complement, complement receptors, and cytokines [3]. Because of the potentially neurotoxic effects of its products, the activation of microglia may be seen as a “double edged sword” that can not only protect but also harm the CNS [8,45]. In the CNS, astrocytes are important for maintaining homeostasis [6,18,21,28,37,48,49] and also have an important role in immune responses. For example, these cells can migrate in response to chemotactic stimuli and gather in areas of injury or inflammation. They can produce complement, complement receptors, and cytokines (especially pro-inflammatory) [10,39].

In this study, we examined differences in microglia and astrocytes, as markers of immune response, and Tau lesions, as indexes of neuronal and neuritic injury, in the
postmortem brains of clinically well characterized control and demented subjects from the Johns Hopkins University Alzheimer’s Disease Research Center (JHU ADRC) and the Baltimore Longitudinal Study of Aging (BLSA) [40]. First, we examined the distribution of immunoreactivity of each antibody, and second, we quantitated these immunoreactivities by measuring the fractional area in the brain region of interest according to the method of Gundersen et al. [17]. We observed a significant increase in the fractional area of reactive microglia associated with the transition from controls to possible AD cases, suggesting that microglia are important in the early pathogenesis of AD. We also found significant increases in the fractional areas of astrocytes and Tau lesions in the neocortex of demented compared to nondemented subjects, suggesting that these changes occur later in the disease and are important for the development of the clinical manifestations of dementia.

2. Materials and methods

2.1. Subjects

Subjects (n = 35) were from the JHU ADRC (n = 10) and the BLSA (n = 25). Cases were selected on the basis of their neuropathological diagnoses according to the CERAD classification [35], as follows. Controls (n = 11) were cognitively normal and their brains were free of SP. Possible AD cases (n = 15) were cognitively normal but their brains showed frequent neocortical neuritic SP on silver stains. Cases similar to these may have been labeled as “high pathology controls” in previous publications by others [29]. Definite AD cases (n = 9) were demented and had frequent neocortical neuritic SP on silver stains. The demographic information, clinical status and neuropathological Braak staging [5] of the subjects are shown in the Table 1.

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<th>Braak</th>
<th>Area Fractional area (%)</th>
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<th>HLA-DR</th>
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* Pathological diagnosis according to CERAD [35].

* The time difference in months between the last MMSE (Mini-Mental State Examination) and death.

* Braak staging [5].
Controls, possible AD cases and definite AD cases did not differ by age. Differences among the three groups were observed, however, for Braak stages and MMSE scores. Specifically, the three groups were significantly different in their distribution by Braak score (P < 0.005). Definite AD cases had significantly lower MMSE scores than controls (P < 0.001) and possible AD cases (P < 0.001). On average, MMSE scores were higher among controls than possible AD cases, but the difference was not statistically significant.

2.2. Diagnostic neuropathology

Brain tissues were fixed in 10% buffered formaldehyde for 2 weeks. Tissue blocks were processed for paraffin embedding, cut at 10 μm, and stained with H&E and the Hirano silver method [52]. The neuropathological diagnosis of AD was formulated according to CERAD recommendations, based on the frequency of neocortical neuritic SP as determined on silver stains [35].

2.3. Clinical evaluations

Clinical evaluations were conducted annually and have been described previously [43]. Briefly, ADRC subjects were seen at the center or, if necessary, in their homes. BLSA participants generally came to the Gerontology Research Center (NIA) every 2 years for the full BLSA protocols [40] and received home visits by the ADRC staff in the year between their regular biennial visits. The evaluations included comprehensive medical history, a complete standardized neurological examinations, and a battery of neuropsychological procedures, including the Mini-Mental State Examination (MMSE) [12] and the Clinical Dementia Rating Scale [4,19]. If a participant was unable to complete an annual visit, they were interviewed by telephone to update the medical history and to administer a Telephone Blessed Information–Memory Concentration Test (T-BIMC) [23]. If a subject showed significant declines on the T-BIMC or other neuropsychological procedures, additional neuropsychological procedures, including the Mini-Mental State Examination (MMSE) [12] and the Clinical Dementia Rating Scale [4,19].

2.4. Immunocytochemistry

Serial tissue sections (10 μm) from paraffin-embedded middle frontal (MFG), (Brodmann’s area 8 or 9) or superior and middle temporal gyri (SMTG), (Brodmann’s area 21 and 22) were examined with single and double immunostains for Aβ, microglia, astrocytes, and Tau proteins using the antibodies described in Table 2. We used the standard avidin–biotin complex procedure (for Aβ and Tau) or the peroxidase–antiperoxidase technique (for microglia and astrocytes) immunostaining. The sections were first treated with methanol/H2O2 for 30 min, to eliminate endogenous peroxidase activity, and rinsed in deionized water for 10 min, followed by microwaving for 7 min. Sections for Aβ immunostaining were also pretreated with concentrated formic acid (80%) for 3 min. Then 3% normal goat serum was used as blocker for 1 h, followed by incubation overnight in the appropriate primary antibody at room temperature. The concentrations for primary antibodies are presented in Table 2. After incubation with primary antibodies, sections were incubated with the appropriate secondary antibodies (ABC-kit, Vector Elite or mouse/rabbit-IgG and mouse/rabbit-PAP) for 1 h in each step. A standard diaminobenzidine (DAB) reaction was used to visualize immunoreactivities. The single-labeled sections were counterstained with hematoxylin. For double immunolabeling HLA-DR/Aβ and GFAP/Aβ, the ABC-kit (Vector Elite) was used to detect the first and second antibodies, with DAB and benzidine dihydrochloride, respectively, as chromagens. For double immunolabeling Tau/HLA-DR, the ABC-kit (Vector Elite) was used to detect the first and second antibodies, with a standard DAB reaction as chromagens.

In a second set of experiments, we used floating sections from the frontal (Brodmann’s area 8 or 9) and temporal (Brodmann’s area 21 or 22) lobes. Tissues were lightly fixed in 4% paraformaldehyde, cryoprotected, frozen, and cut at 40 μm on a sliding microtome. Floating sections were single or double immunostained with antibodies for Aβ, microglia, and astrocytes. The standard avidin–biotin complex procedure was used in floating sections with the same antibodies as described in the paraffin procedure. Sections were pretreated with methanol/H2O2 for 20 min and washed in Tris buffered saline (TBS, 3× 10 min). Sections for Aβ immunostaining were pretreated with 15% formic acid for 5 min, blocked in 4% normal goat serum and 0.1% Triton X-100 in TBS for 1 h and incubated with primary antibodies for 24 h at 4 °C. The concentrations for primary antibodies were: Aβ 1–28, 1:1000; HLA-DR 1:500; and GFAP 1:2000. After incubation with primary antibodies, sections were incubated with the appropriate secondary antibodies (ABC-kit, Vector Elite) at room temperature, each step for 1 h.

Table 2

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<tr>
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<td>Microglia</td>
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<td>GFAP</td>
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<td>Dako</td>
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<tr>
<td>Tau (Tau-2)</td>
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The concentrations for primary antibodies in ICC (paraffin) studies of human brain.
Before exposure to the second primary antibody, tissues were blocked in 4% normal goat serum (without Triton X-100) in TBS for 30 min at room temperature. The tissue sections were then incubated with the second primary antibody overnight at 4°C. The second antigen was detected with the ABC-kit (incubation for 1 h at room temperature in both steps) with benzidine dihydrochloride as chromagen and 0.01 M sodium acetate as buffer (pH 6.0). In each run, negative controls, with no primary antibody, were used to assess specificity.

2.5. Qualitative analyses of senile plaques

The qualitative analyses utilized both single and double immunostains. We compared the pathological changes between nondemented and demented subjects, in both SPs and in the intervening neuropil. We also examined the possible co-localization of Tau immunoreactivity with microglia and/or astrocytes.

2.6. Quantitative analyses of immunoreactivities

Quantitative analyses were conducted on single immunostains. In order to measure the amount of immunoreactivity (IR) for each antibody, the fractional area (FA) of the IR was measured by the method of Gundersen et al. [17]. The region of cortex analyzed was delineated with the help of a microscope equipped with stepping motors controlled by Stereo Investigator Software (MicroBrightfield, Inc., Vermont). A group of 20 points was placed in a systematic random position, at 1000 μm intervals, within the boundary of each region. The sum of the points falling over structures of interest (e.g. microglia) was divided by the total number of grid points sampled to estimate the fraction of the area of the region occupied by a particular type of cell. According to the Delesse principle (Gundersen et al.), the FA is equal to the fraction of the volume (Vp) occupied by the cell type being quantified. This method measures the percentage of the area of interest (in this case, the cerebral cortex) that is

![Box plots of the distribution of samples by disease status (i.e. control, possible AD, definite AD) for Aβ, GFAP, HLA-DR, Tau. The box area indicates the interquartile range, the line in the box indicates the median value, and the dashed lines indicate the 90% range.](image)
immunoreactive for a specific antibody. First, we outlined an area (100 mm$^2$) of cortical gray matter (either MFG or SMTG, Table 1) that included all neuronal layers (from pia to the boundary with the white matter). Then, starting from a random site, the computer provided a systematic sampling of the outlined field (which was divided randomly into 1000 $\mu$m × 1000 $\mu$m), and at each selected site it projected a counting frame (130 $\mu$m × 100 $\mu$m) and a superimposed counting grid composed of small equidistant crosses. We counted all the crosses that fell on the cerebral cortex (total number of crosses) and the crosses that coincided with immunoreactivity (IR) “hits”. IR was counted whether or not it was associated with SP. The sum of hits for all counting frames divided by the total number of crosses is the FA. A change in this parameter can involve changes in the number or size of the cells, or in both. Based on this data it is impossible to determine which factor has changed. We counted on average the following number of hits/points for different immunostains; with HLA-DR antibody in AD 40/2768, in AD possible 28/3518, and in controls 23/3165; with GFAP antibody in AD 162/1645, in AD possible 97/1994 and in controls 105/1933; with A$\beta$ antibody in AD 98/1919, in AD possible 65/2266, and in controls 5.6/2355; and with Tau antibody in AD 43/3457, in AD possible 0/2200, and in controls 0/2300. This procedure was repeated for each antibody in the same cortical area. The individual conducting the measurements was blind to diagnostic categories.

2.7. Co-localization of A$\beta$ and microglia

To examine the co-localization of A$\beta$ cores and microglia in SP, we randomly selected five cases of possible AD and five of definite AD. On double immunostains, we examined and counted 150 SPs in each case by using the Stereo Investigator System. Each SP was then classified as: without core or microglia, without core but with microglia, or with core and with microglia.

2.8. Statistical analyses

Summary statistics (mean and 95% confidence intervals) were derived for each of the four neuropathological markers among controls, possible AD cases, and definite AD cases. Statistical differences in the fractional area of A$\beta$ deposits, microglial and astrocytic activity, and Tau among the three study groups were evaluated using the Wilcoxon Signed Test. This is a rank order nonparametric test suitable for small sample sizes or when values are not normally distributed. For each comparison between groups of size $n_1$ and $n_2$ each value is assigned a rank order between 1 and $n_1 + n_2$. Significance testing is based on a comparison of the sum of the rank scores derived for each group. All groups were compared to each other (i.e. four markers and three possible comparisons) on each marker.

3. Results

3.1. Qualitative observations

For the qualitative analyses we studied single immunostains for A$\beta$ (Figs. 1 and 2), HLA-DR (Fig. 3), GFAP (Fig. 4) and Tau (Fig. 5) on each subject. To examine the composition of SPs, we used double immunostains for A$\beta$/HLA-DR (Figs. 6 and 7B) and A$\beta$/GFAP (Fig. 7A). The composition of SP, as examined by their IR with the various antibodies, was heterogeneous. Some SP were IR only for A$\beta$ but not for astrocytes or microglia. The majority of these SP were of the diffuse type and most were found among possible AD cases. Activated microglia, detected by HLA-DR IR, were common in the SPs of definite AD as well as in possible AD, and they appeared to be associated with the core of the plaque in both groups (Figs. 6 and 7B). Comparing controls (no SPs, normal cognition) (Fig. 3A) and possible AD cases (SPs, normal cognition) (Fig. 3B), reactive microglia increased markedly in the latter group in association with SPs and in the neuropil. Astrocytes, demonstrated by GFAP IR, were typically located in the periphery of SPs in cases of possible (Fig. 4B) and definite AD (Figs. 4C and 7A); but in the latter were also abundant in the neuropil. Again, Tau IR revealed the sharpest distinction between possible (Fig. 5A) and definite AD (Fig. 5B–D) cases, as Tau immunoreactivity...
was observed only in definite AD cases, both within SP (Fig. 5D) and in the neuropil (Tau threads in Fig. 5B), (neurofibrillary tangles in Fig. 5C). In definite AD cases, we detected Tau positive microglia especially in association with cores (Fig. 9).

3.2. Quantitative results

Measurement of fractional areas (mean ± S.E.M.%): Aβ FA was negligible, 0.2 ± 0.06%, in controls, 2.7 ± 0.5% in possible AD, and 5.2 ± 0.9% in definite AD, showing a significant differences among groups. HLA-DR reactivity showed a gradual increase, from 0.8 ± 0.3% in controls to 1.2±0.3% in possible AD and 1.4±0.3% in definite AD. The difference in HLA-DR FA between controls and possible AD cases was significant (P < 0.05). In contrast, FA for GFAP was only slightly different between controls, 5 ± 1% and possible AD, 4.2 ± 0.6%, but significantly higher in definite AD, 9.3 ± 1.8%, compared to possible AD (P < 0.01). Tau IR revealed the sharpest distinction between nondemented and demented cases (P < 0.001), as Tau IR was observed only in the brains of subjects with definite AD, 1.3 ± 0.3%. The box plots of the distribution of samples by disease status and FA for specific antibodies are displayed in Fig. 1. In the examination of co-localization of SP-cores and microglia, the cores were always associated with reactive microglia, although the proportion of the different types of plaques was different in possible AD compared to definite AD, as

Fig. 3. HLA-DR immunostaining in human neocortex: (A) control; (B) possible AD; (C and D) definite AD (at different magnifications).

Fig. 4. GFAP immunostaining in human neocortex: (A) control subject with astrocytes around small blood vessels; (B) possible AD case with astrocytes surrounding a SP; (C) definite AD with abundant astrocytes around senile plaque and in neuropil.
Fig. 5. Tau immunostaining in neocortex: (A) no staining in possible AD; (B) neuropil Tau threads in definite AD; (C) neurofibrillary tangle in definite AD; (D) senile plaque in definite AD.

Fig. 6. Aβ and microglia double-immunostains. Floating tissue sections (40 μm) from neocortex in AD case. Bdhc (blue) staining for Aβ 1–28 (Athena antibody) and Dab (brown) staining for microglia (HLA-DR): (A) note microglial cells (brown) inside senile plaques; (B) Aβ deposits (blue) next to blood vessels; (C) microglial cells (brown) along and around blood vessels; (D) Aβ deposits (blue) and microglial cells (brown) next to blood vessel.

shown in Fig. 8. In the cases of definite AD, 42.3 ± 10.7% of the plaques had no core or microglia, 34.8 ± 5% had only microglia, and 22.9 ± 6.7% had cores and microglia. In possible AD cases, 51.2 ± 14% of the plaques had neither core nor microglia, 29.2 ± 7.9% had only microglia and 19.6 ± 6.6% had cores and microglia. These differences did not reach statistical significance with this small sample size (5 AD and 5 AD possible). Notably, we did not find plaque cores without microglia in cases of either possible or definite AD (Figs. 6 and 7).

4. Discussion

4.1. Subjects

The subjects in this study were clinically well characterized and 45.7% of them had been examined within a year before death and 74.3% within 2 years (Table 1). This short interval between the last clinical evaluation and autopsy enhances the value of the clinical pathological correlations examined in this study. It is also important to point out that
the demented group (with definite AD) had a wide range of MMSE (from 0 to 26) and Braak stages (from II to VI). The inclusion of subjects in early stages of dementia may reduce the differences in pathological markers between the demented and nondemented group.

4.2. Aβ deposition

Aβ is normally expressed in neurons and nonneural cells, yet its function remains unclear [38,41]. Under normal conditions, the metabolism of Aβ does not result in its accumulation, but during aging many humans develop Aβ deposits in brain parenchyma and vessels [14]. In our observations, the FA for Aβ, also known as Aβ load, varied significantly among the control group and the possible and definite AD groups. This is not surprising because the brains for this study were selected according to neuropathological diagnoses and, therefore, on the basis of the amount of SP in the neocortex. Our observations suggest that Aβ deposition is an early event in the pathogenesis of AD; however, the lack of dementia in subjects with moderate Aβ deposition, i.e. the possible AD group, suggests that this pathological event is necessary but not sufficient for the development of dementia in AD.

4.3. Microglia

It was of interest that the FA for microglia increased significantly in possible and definite AD cases compared to controls. Because this increase in microglia was associated with the appearance of Aβ in possible AD cases, we speculate that microglia may be increased as a reaction to Aβ and constitute an important factor in the early stages of AD. We also observed that microglia were present within the Aβ deposits and, in particular, were associated with the core of SPs. This strong association of microglia with the core of SPs suggests a role for these cells in the processing of Aβ. Microglia may phagocytize β-amyloid protein [15,20,36] and also the remains of dystrophic neurites, as suggested by the presence of Tau IR in microglial cells. At the same time, activated microglia may secrete neurotoxic products [8,26,46]. A previous study examined the brains of non-demented individuals with substantial Alzheimer’s lesions and referred to them as high pathology controls (HPC) [29]. This study found that HPC brains did not have increased microglia as detected by LN3 immunoreactivity. By contrast, we have demonstrated that microglia, as detected by HLA-DR immunostain, is increased in subjects comparable to HPC. This difference may be explained by differences in the stage of the dementia in the subjects, or by differences in the expression of microglial epitopes detected by LN3 or HLA-DR. An important question is whether changes in microglia may reflect different pre-mortem and agonal circumstances. Although pre-mortem and agonal stage information is not available in our subjects, in our experience a larger proportion of nondemented individuals, i.e. controls and possible AD, die suddenly compared with demented patients, i.e. definite AD. It is believed that protracted agonal states, common in bedridden patients with advanced AD, may cause increased activation of brain microglia. However, since the difference in microglia in our study was between controls and possible AD subjects, all of them fully active, we believe that this difference represents the evolution of the disease and is not secondary to agonal stages.

4.4. Astrocytes

We observed a statistically significant association between dementia and astrocytic activation (i.e. FA of GFAP) in possible versus definite AD, but not between controls and possible AD. The observed increase in the FA of GFAP can result from a change in the number of astrocytes, in the change in the volume of astrocytes or in both. In the brains
of demented individuals, GFAP showed doubling of IR, reflecting the presence of astrocytes not only in SPs but in the intervening neuropil. The difference in GFAP IR between controls and AD possible cases was not significant, suggesting that astrocytic reaction is a late event in the response to Aβ deposition, and that it may follow microglial activation. When stimulated by Aβ, reactive astrocytes may produce neurotoxic chemokines and cytokines. Taken together, our observations suggest that the development of cognitive decline in AD is associated with the proliferation and activation of astrocytes in the cerebral cortex. The peripheral distribution of astrocytes around Aβ deposits suggests a role in containing or circumscribing the abnormal protein, a well-known function of astrocytes in response to injury.

4.5. Tau

A central neuropathological feature of AD is the presence of neurofibrillary tangles, which are composed of hyperphosphorylated Tau protein [27]. Tau immunoreactivity (Tau-2) revealed the sharpest difference between nondemented and demented cases, as it was observed only in the neuropil of definite AD. However, earlier Lue et al. [29], have reported nondemented elderly with profuse neurofibrillary tangles i.e. high pathology controls detected by Thioflavine. The sharp contrast seen in our study in Tau-2 immunostain between demented and nondemented individuals might reflect low sensitivity of the Tau-2 antibody compared to silver stains and thioflavine. However, the significant difference in Tau-2 immunoreactivity between demented and nondemented individuals raises the question whether the conformational change in the epitope of Tau corresponding to amino acids 95–108 [50] plays a role in the development of dementia. We also detected Tau-2 immunoreactivity in microglial cells, particularly in association with the plaques with core, but also in the neuropil (Fig. 9). Previously, it has been speculated whether this is due to a cross-reactive antigen or Tau in microglia [44]. However, in our experiment we detected HLA-DR positive microglia with and without Tau-2 immunoreactivity in the same case and in the same microscopic field, which speaks for specific binding of Tau in microglia.

Several conclusions may be drawn from this study. First, Aβ deposits in AD appear necessary but not sufficient to cause dementia, as they were observed in demented and nondemented subjects. However, the increasing Aβ load seems to play a role possibly in triggering additional pathogenic factors (microglia, astrocytes, etc.) and driving the disease progress. Second, increased reactive microglia is associated with the increased Aβ load and transition from control (no Aβ) to possible AD (Aβ+), suggesting a role for these cells in the early pathogenesis of the disease. Third, the significant association between cognitive decline and proliferation and activation of astrocytes suggests that these cells may play a role in the late stage of AD. The location of astrocytes mainly in the periphery of SP may reflect the roles of these cells in immune-defense. Fourth, Tau-2 immunoreactivity appears as a strong morphological correlate of dementia, existing only in definite AD cases.

In summary, it appears that Aβ deposition is an early event in the pathogenesis of AD, but is insufficient to cause dementia. Our observations suggest that the proliferation and activation of microglia and astrocytes constitute secondary steps in the pathogenesis of dementia in AD.
Furthermore, it appears possible that the microglial changes may precede the astrocytic proliferation and activation associated with dementia. Although these changes of microglia and astrocytes appear to be down-stream to Aβ deposition, they still represent reasonable targets for treatment.

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