Brief Definitive Report

AUTOANTIBODIES TO NEUROFIBRILLARY TANGLES AND BRAIN TISSUE IN ALZHEIMER'S DISEASE

Establishment of Epstein-Barr Virus–transformed Antibody-producing Cell Lines

BY FELICIA GASKIN, BEVERLY S. KINGSLEY, AND SHU MAN FU

From the Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

Alzheimer's disease (AD) is a major form of dementia. The pathological hallmark is a marked increase of neuritic plaques and neurofibrillary tangles (NFT) in certain regions of the AD brain. Despite considerable advances, the etiology and pathogenesis of AD remain unknown (reviewed in reference 1). Although autoimmunity and other immunological factors have been proposed to play certain roles, previous studies to demonstrate serum antibrain and other relevant antibodies have been complicated by the presence of high titers of other autoantibodies such as antinuclear antibodies (2). Our approach to circumvent this difficulty is to derive multiple B cell lines by Epstein-Barr viral (EBV) transformation of a limited number of B cells from the blood of patients with clinically diagnosed AD and age-matched controls. The present studies describe our findings, by immunocytochemical techniques, that certain EBV-transformed cell lines secrete autoantibodies reactive with NFT and neuronal tissue.

Materials and Methods

Derivation of EBV-transformed B Cell Lines. Non-T cells were isolated according to Fu et al. (3) from the peripheral blood of five patients with clinically diagnosed AD, ages 59, 61, 66, 68, and 75, respectively and six normal age-matched controls ages, 60, 61, 66, 66, 71, and 72, respectively. They were seeded at $1.5–3.0 \times 10^3$ cells/well in RPMI 1640, 10% FCS, with 10^4 allogeneic mononuclear cells irradiated with 3,000 rad as feeder cells, in 96-well plates. EBV-containing B95-8 cell line supernatant was added and culture medium was replaced weekly. Cell lines were considered to be established when the cell cultures were expanded to 7 ml at 5×10^5 cells/ml. They were cryopreserved and the supernatants were analyzed for antibodies (Ab) of desired reactivity. Passive hemagglutination inhibition (3) showed that most of the supernatants secreted IgM at 0.5–5 μg/ml. The cell lines were either monoclonal or oligoclonal as indicated by the presence of a single class of light chains.

Immunocytochemistry. SDS-treated isolated NFT were prepared by the long procedure of Iqbal et al. (4) using frontal and temporal cortex from two autopsied brains with a neuropathological confirmation of AD. 2 μl of isolated NFT containing 50–100 NFT

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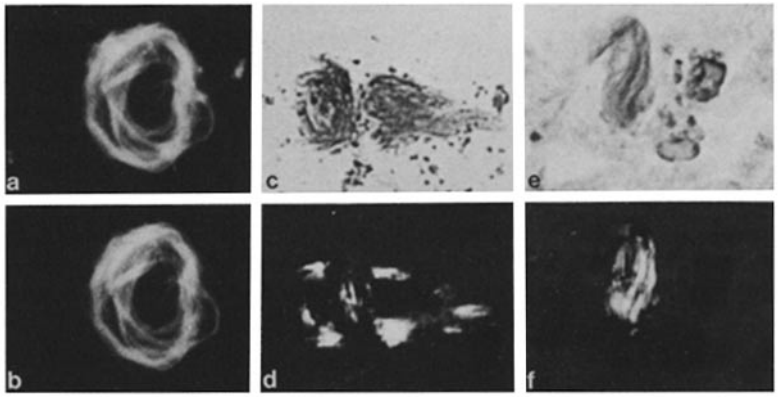

FIGURE 1. Staining of NFT by autoantibodies from patients with clinically diagnosed AD. (*a*) JGR 29 labels an isolated NFT as detected by rhodamine-conjugated goat anti-human Ig (red fluorescence). (*b*) The same NFT was counterstained with thioflavine S, which emits green fluorescence (× 900). (*c*) KKN 7 stains isolated NFT as detected by an ABC method. (*d*) The two NFT were identified by their birefringence with Congo red (× 600). (*e*) KKN 7 stains NFT in situ (temporal cortex) as well as other neuronal elements. (*f*) In this section, the NFT is identified by Congo red (× 600).

were air dried for 15 min on gelatin-coated slides and fixed for 6 min in methanol at −18°C. After washing with RPMI or PBS, the NFT were incubated for 2 h at room temperature, with supernatants from EBV-transformed B cell lines. The bound Ab was detected with rhodamine-conjugated goat anti-human Ig with specificities to μ, γ, α, and Fab (5). The NFT were further treated with 0.001% thioflavine S in 10% buffered formalin for 6 min to identify NFT by green fluorescence (5). The supernatants were also screened by immunofluorescence against methanol-fixed cells from the following cell lines: HeLa, a human fibroblast line (GM3652), and a human neuroblastoma cell line (GM3320C). Supernatants were also characterized by the avidin-biotin-complex (ABC) immunoperoxidase method (using IgG and IgM reagents and methodology from Vector Laboratories, Burlingame, CA, and methanol fixation) for reaction with isoated NFT and frontal and temporal cortex on 5 μm cryostat sections from normal and AD brains. With the ABC method, NFT were identified by their green birefringence with Congo red.

Results

Higher Frequencies of NFT-reactive Ig-secreting Cell Lines from AD Patients. EBV-transformed B cell lines were established from five AD patients and six normal age-matched controls. Their supernatants were tested for reactivity with SDS-treated isolated NFT by immunofluorescence. NFT were identified by staining with thioflavine S, which emits green fluorescence under UV. A typical double-labeled NFT is shown in Fig. 1, *a* and *b*. The reactivities of several anti-NFT Ab were confirmed using the ABC antibody method and Congo red binding to NFT, which results in green birefringence under polarized light (Fig. 1, *c* and *d*). NFT-reactive supernatants were identified from cell lines derived from all five AD patients. The frequencies of reactivity were 14/115, 4/113, 1/80, 4/68, and 4/50, respectively, with a combined frequency of 27 out of 426, or 6.3%. In contrast, the frequencies of anti-NFT reactivity of 250 cell lines from six age-matched controls screened thus far were 1/50, 1/50, 1/50, 1/25, 0/50, and 0/25, for a combined frequency of 4 out of 250 or 1.6% (Table I). These and additional supernatants were studied with HeLa cells. A higher percentage of

TABLE I

Percentages of EBV-transformed B Cell Line Supernatants Derived from AD and Age-matched Controls that React with NFT, Temporal Cortex, and Cultured HeLa Cells

	Isolated NFT	NFT in temporal cortex	Temporal cortex	HeLa cells
AD	6.3 (27/426)	2.3 (8/350)	6.8 (39/575)	18.2 (110/605)
Controls	1.6 (4/250)	0.8 (2/243)	6.2 (15/243)	11.9 (60/504)

TABLE II

Staining Patterns of Supernatants from Representative Cell Lines from AD Patients and Age-matched Controls

Cell lines	Isolated NFT*	Temporal cortex‡		HeLa, fibroblast, and neuroblastoma cell lines
		AD	Normal	
AD patients	+++	Neurons, NFT	Neurons	Nucleoli, cytoplasm
KKN 7 (IgMκ)	+	Neurons, NFT	Neurons	Nuclei
KKN 92 (IgMλ)	+ to +++	Astrocytes, NFT	—	—
KKN 122 (IgMκ)	+++	Neurons, NFT	Neurons	Filaments
JGR 29 (IgMκ)	++	Neurons, NFT	Neurons	Cytoplasm
JGR 38 (IgMλ)	++	Neurons, glia, NFT	Neurons, glia	Filaments
MHI 16 (IgMκ)				
Controls	+	Neurons	Neurons	Cytoplasm
BRH 17 IgMλ)	+	—	—	—
CAN 19 (IgGλ)	−	Neurons, glia	Neruons, glia	Filaments
CNE 19 (IgMκ)				

* Immunofluorescence and double labeled with thioflavine S. Staining was ranked − for negative, and the number of + signs are proportional to the intensity of staining.
‡ ABC method. NFT were identified with Congo red.

HeLa reactive supernatants was obtained from the AD group (18.2 vs. 11.9%). When these supernatants were tested by the ABC method on temporal cortex sections from autopsied AD brains, comparable percentages (6.8 vs. 6.2%) of the reactive supernatants from two groups were obtained. However, a higher percentage (2.3 vs. 0.8%) of supernatants of cell lines from the AD group were shown to stain NFT in situ, as demonstrated in Fig. 1, *e* and *f*.

Over 300 EBV-transformed cell lines were established from three younger patients with systemic lupus. Among 65 cell lines shown to secrete Ab reactive with HeLa cells, only one stained SDS-treated, isolated NFT. The frequency for anti-NFT is low in this selected group, although the frequency for anti-HeLa activity was similar to those of AD patients.

Different Staining Patterns by Anti-NFT Antibodies. The NFT-reactive supernatants of cell lines from both AD and control groups were studied repeatedly on SDS-treated isolated NFT, temporal cortex sections from three AD and two normal autopsied brains, and HeLa, fibroblast, and neuroblastoma cell lines. However, several different staining patterns emerged. The results of selected cell lines are summarized in Table II.

The reactivity of KKN 122 is of special interest. It stained SDS-treated isolated NFT. It reacted with NFT and astrocytes in the temporal as well as frontal cortexes of three autopsied AD brains. Thus far, it did not stain sections of normal brains and cells of the three cell lines. The remaining five of the six

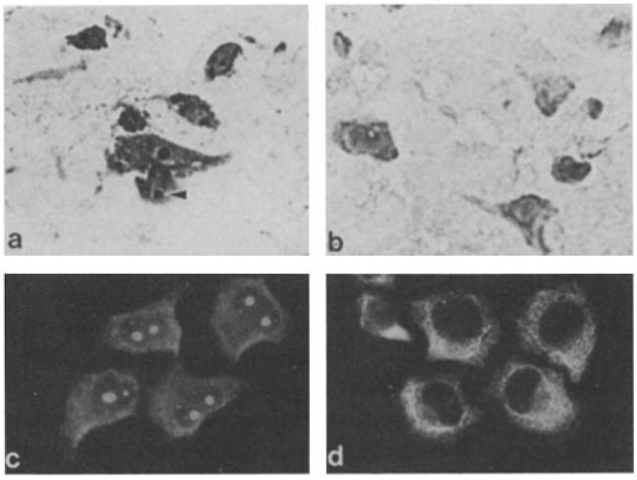

FIGURE 2. Different staining patterns of KKN 7 and JGR 29 on normal brain sections and with cultured HeLa cells. KKN 7 and JGR 29 labeling of temporal cortex is shown in *a* and *b*, respectively (× 480). Both autoantibodies react with neurons. KKN 7 staining of bright nuclear spots is prominent (*arrow*). (*c*) KKN 7 staining of HeLa cells (× 375). In addition to cytoplasmic staining, nucleolar staining is evident. (*d*) JGR 29 stains the cytoplasm of HeLa cells in a filamentous pattern (× 375).

presented anti-NFT supernatants from AD patients stained NFT in situ, neurons in both AD and normal brains, and all three cell lines. One of them stained glia as well. However, their staining patterns differed from each other. Fig. 2 shows the staining patterns of KKN 7 and JGR 29. Few anti-NFT supernatants were identified from the control group. In general, they were weaker in staining intensity. Of the three presented cell lines from the control group, supernatants with staining patterns similar to those of BRH 17 and CNE 19 were identified in the AD group. Thus far, the staining pattern of CAN 19 is unique.

Discussion

In this study, anti-NFT Ab–secreting B cell lines have been shown to be readily established from AD patients and age-matched controls. Higher frequencies were seen in cell lines from the AD group. The finding of an even lower frequency of this reactivity in a group of similarly transformed cell lines with high frequencies of autoantibodies from much younger patients with systemic lupus supports the interpretation that the observed higher frequency in the AD group is likely to relate to the disease state as well as the ages of the patients. The higher frequency of anti-NFT Ab–secreting cell lines in AD patients reflects the higher frequency of circulating B cells capable of making such Ab. The expansion of this B cell pool might be a part of an immune response to inflammation and damage of neuronal tissue. The leakage of antigens (Ag) from the nervous system to the systemic circulation might be responsible for the initiation of this response. An example of this has been documented by the demonstration of increased serum Ab titers against glycosphingolipids in Multiple Sclerosis patients and patients recovered from strokes and head traumas (6). However, in AD, this type of immune response may not be strong enough to allow easy detection. The

approach taken in this study would provide a method for the detection of such a weak immune response. In addition, the possibility of applying the present approach to other degenerative neurological disorders should be explored.

An interesting supernatant is KKN 122. It stained isolated NFT as well as NFT in situ and astrocytes of AD temporal and frontal cortex. Thus far, it did not stain the three cell lines and two normal non-AD brains. Additional studies with autopsied normal and AD brains are needed to ensure the specificity of this Ab. The staining of astrocytes is of considerable interest because of the marked increase in astrocytes in affected regions in AD brains (7). A monoclonal Ab (mAb) prepared using AD brain tissue as an Ag has been reported (8) to identify an Alz-50 Ag present on neurons containing NFT in AD brain. The lack of staining of neurons by KKN 122 indicates that the reactive Ag differs from Alz-50. Recently, we have identified an additional cell line from KKN to have similar reactivity as KKN 122. The possibility that additional cell lines secreting Ab specific for Alz-50 needs further exploration.

The majority of the anti-NFT Ab stained neurons as well as cells of different origins. The B cell lines have been established by EBV transformation with 1,500–3,000 non-T cells seeded in the initial cultures. The use of these limited numbers of non-T cells ensures that the established cell lines are either monoclonal or oligoclonal. The finding of a single class of light chains in the majority (>90%) of these supernatants adds support to this assumption. In addition, two cell lines, one of which is KKN 7, have been cloned by limiting dilution. >60% of the clones gave staining patterns similar to that of the parental line. The other clones did not stain the cells or tissue of interest. Thus, the staining of neurons, glia, and cell lines observed in these studies was likely due to a single Ab. The differing reactivities with various cellular elements would indicate that the composition of a NFT is complex. It remains to be determined whether the reactive Ag are similar to those identified by polyclonal or mAb to NFT or cytoskeletal proteins (reviewed in references 1 and 9). Recently, serum Ab to NFT and brain tissue have been reported in AD patients (10–12). Our attempts to specifically stain NFT and brain tissue using serums have not been successful due to high background staining of control serums. Newer methodology is needed to demonstrate the presence of serum Ab with similar specificities as those secreted by EBV-transformed cell lines.

Summary

Multiple EBV-transformed B cell lines were established from five patients with a clinical diagnosis of Alzheimer's disease (AD) and six age-matched controls. The supernatants were screened for antibody activity against SDS-treated isolated neurofibrillary tangles (NFT). Reactive supernatants were identified from both the AD and control group. The frequencies of anti-NFT antibody–secreting lines were 6.3 and 1.6% for the AD and the control groups, respectively. A proportion of these supernatants also stained NFT in situ and neurons and/or glia in sections of the frontal and the temporal cortexes of autopsied AD and normal brains, as well as cells from three cell lines (HeLa, fibroblast, and neuroblastoma). Several patterns of staining were revealed by these supernatants, indicating different reactive antigens. One supernatant stained NFT and astro-

cytes in sections from AD brains. It did not stain sections from two normal brains. This cell line is the result of the immortalization of a circulating B cell making antibody specific for an antigen in AD. The present approach may provide new insights in the pathogenesis of AD.

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