Differential Expression of Nucleoskeleton- and Cytoskeleton-associated Proteins in Burkitt Lymphoma-derived and Epstein-Barr Virus-immortalized Lymphoblastoid Cell Lines

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Abstract
Mouse monoclonal antibodies raised against nuclear bodies isolated from an EBV-immortalized lymphoblastoid cell line (LCL) known to contain several viral and cellular proteins (Jiang et al., Exp. Cell Res., 197: 314–318, 1991; Szekely et al., J. Gen. Virol., 76: 2423–2432, 1995; Szekely et al., J. Virol., 70: 2562–2568, 1996). Seventy six clones gave detectable immunofluorescence staining on LCLs. Five independent monoclonal antibodies detected a group of apparently novel, high M₉ proteins that shared common features of subcellular distribution. In LCLs, these proteins were preferentially associated with vimentin filaments in the cytoplasm and with distinct nuclear foci. The appearance of the latter differed from the premyelocytic leukemia-associated protein, EBV nuclear antigen #5, and retinoblastoma-protein-positive bodies that were used for immunization. They seemed to be connected to the cytoplasmic filaments through thin fibrillar nuclear structures. In mitotic cells, these complex structures rearranged into a perichromosomal basket that was associated with vimentin filaments. The target proteins, operationally designated as proteins associated with nuclear dots and cytoplasmic filaments (pNDCFs), were not present in resting human B cells or were expressed at a low level. The level increased considerably after EBV infection or mitogenic stimulation by interleukin 4 and anti-CD40 antibodies. In Burkitt lymphoma (BL) type I lines phenotypically representative of the in vivo tumors, the pNDCFs were either absent or exclusively localized to the nucleus, usually to well-defined nuclear foci. EBV-positive type I BLs often shift to a more LCL-like (type III) phenotype during prolonged in vitro propagation. Type I cells express only EBV nuclear antigen 1 and the surface markers CD10 and CD77, whereas type III express all nine growth-associated EBV-encoded proteins and a gamut of B-cell activation markers. Most of the type III BL cell lines contained increased amounts of pNDCFs bound to cytoplasmic filaments, as seen in the LCLs. We propose that the expression of vimentin-associated pNDCFs should be included in the definition of type III BL phenotype.

Introduction
EBV transforms resting human B cells into immortalized LCLs. The infection of B cells leads to increased expression of a plethora of cellular genes. Their products include immunoglobulins; cell surface molecules like CD21, CD23, and CD44; adhesion molecules intercellular adhesion molecule 1, LFA-1 β-chain (CD18), and LFA-3 (CD58); structural protein vimentin (1); tyrosine kinase c-fgr (2, 3); a variety of cytokines; nuclear growth-regulatory molecules such as c-myc (4), b-myb (5), p53 and RB proteins (6), cyclin D2 (5, 7), cyclin D3 (8), cyclin E (5), and bcl-2 (9); and predicted protein G-coupled peptide receptors EBI-1 and EBI-2 as well as genes for cathepsin H, annexin VI (p68), serglycin proteoglycan core protein, CD44, and the myristylated alanine-rich protein kinase C substrate (10).

Human B cells are immortalized by the concerted action of at least six of nine EBV-encoded, latency-associated proteins (EBNA-1, -2, -3, -5, and -6 and latent membrane protein 1 (11–14). Two of them, EBNA-2 and EBNA-5, are expressed already at a very early phase of infection (4). They drive the infected cell from G₀ to G₁ in parallel with the induction of cyclin D2 (7). Previously, we have detected nuclear bodies in EBV-transformed LCLs containing EBNA-5, RB, heat-shock protein Hsp70, and PML protein (15–18).

In the course of these studies, we have also found that most of the EBNA-5 is strongly associated with the nuclear matrix. Cell fractionation by sequential elution showed that even 8 M urea was unable to release the matrix-bound EBNA-5, whereas boiling in SDS completely disrupted the association (16). Complexing partners of matrix-bound proteins cannot be studied with traditional immunoprecipita-

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The abbreviations used are: LCL, lymphoblastoid cell line; mAb, monoclonal antibody; BL, Burkitt’s lymphoma; PML, promyelocytic leukemia; pNDCF, proteins associated with nuclear dots and cytoplasmic filaments; IL, interleukin; IF, intermediate filament; EBNA, EBV nuclear antigen; RB, retinoblastoma protein; IFAP, intermediate filament-associated protein.
Polyclonal serum

anti-PML

overlap

Fig. 1. Top row: A, immunofluorescence staining of human fibroblasts with the polyclonal serum of the BALB/c mouse immunized with the EBNA-5-associated nucleoskeletal protein aggregates (yellow, FITC fluorescence). B, PML staining of the same cell (red, Texas Red fluorescence). C, overlap of the two images shows that the polyclonal serum, among other structures, recognizes components of the PML-positive blobs (white, overlapping areas). Bottom rows: A–E, the five different categories of the resultant mAbs are summarized in Table 1. (Yellow, immunofluorescence staining with FITC; blue, Hoechst 33258).

tion methods due to their insolubility in nondenaturing lysis buffers. To identify other proteins that share subnuclear distribution with EBNA-5, we developed a method to isolate protein complexes from organic solvent-fixed and mechanically disrupted cells using anti-EBNA-5 mAbs immobilized on the surface of magnetic beads. These complexes were used as antigens to raise mAbs against the individual components. The resulting polyclonal antibodies and mAbs were
found to be mainly directed against nuclear matrix-associated proteins.

In the present paper, we describe a group of mAbs obtained in the course of these experiments that react with high molecular weight proteins that do not seem to have been identified previously. Operationally designated as pNDCFs, these proteins showed differentiation-dependent expression in mouse embryos. They were not expressed or were only minimally expressed in resting human B cells but were readily induced by EBV and anti-CD40 plus IL-4, respectively. They also showed a marked increase in expression and a change in subcellular distribution during the in vitro phenotypic shift of BLs toward a more LCL-like phenotype.

Results

Antigen Preparation and Immunization. Our experiments were designed to identify EBNA-5-bound proteins. Due to the strong interaction between EBNA-5 and the nuclear skeleton and the insolubility of such structures in mild lysing buffers, coimmunoprecipitation experiments were not readily feasible. Therefore, we designed a simple method that generates protein aggregates that can survive mechanical disruption by sonication without covalent cross-linking of the individual components. We found that organic solvent (e.g., methanol-acetone)-fixed cells can be broken up by sonication at relatively low energy levels to an almost homogenous suspension of very small particles. Subfractions that have a certain specificity can be captured with magnetic beads loaded with a mAb with the desired specificity. The validity of the method was tested in the following way. Magnetic beads loaded with either anti-EBNA-2 (PE2) or anti-EBNA-5 (JF146) mAbs were incubated with lysates of the IB4 LCL. We have previously shown that EBNA-5 but not EBNA-2 colocalizes with the PML protein in distinct nuclear foci in the IB4 cell line. After incubation with IB4 lysates, the mAb-loaded magnetic particles were immunostained with a rabbit polyclonal antibody against PML. Only the anti-EBNA-5 mAb-coated beads were found to bind PML containing aggregates. The anti-EBNA-5-captured protein aggregates were used to raise mAbs as described in "Materials and Methods." The polyclonal sera of the immunized mice stained the nucleus preferentially. The staining patterns were composed of numerous nuclear dots, larger nuclear bodies, and lamina-like structures in the nuclear periphery. Double immunofluorescence staining with PML antibodies showed the nuclear bodies detected by the mouse polyclonal sera colocalized with the PML-positive nuclear bodies in human fibroblasts (Fig. 1).

Analysis of Hybridomas. The fusion from the best reactive mouse (DM) generated more than 600 hybridoma clones. A total of 146 clones showed high immunoglobulin secretion and were further tested by immunostaining IB4 cells. Seventy-six hybridoma clones gave detectable fluorescent staining. Sixty-four of them stained the nucleus and recognized mainly distinct nuclear foci. Fifty-six were expanded into stable growing cell lines. Fifty of them produced antibodies against nuclear antigens. The antibodies were divided into five categories (A–E) on the basis of staining pattern as summarized in Table 1. Fig. 1 exemplifies the different categories. Group C was selected for immediate further analysis because the combination of the nuclear and cytoplasmic reactivity pattern seemed particularly interesting. Five of seven hybridoma clones (DM-1B5, DM-4A6, DM-5C6, DM-6A2, and DM-10H2) became stable immunoglobulin producers and were used to raise ascites in SCID mice for additional studies. The distribution of the reactive proteins, designated as pNDCFs, indicated that they may represent a previously unidentified group of proteins that may form links between distinct membrane regions and defined subnuclear domains.

Intracellular Distribution of pNDCFs. In IB4 cells, anti-pNDCFs detected well-circumscribed nuclear foci, prominent cytoplasmic filaments, and weak nuclear filamentous structures (Fig. 2). One of the mAbs (DM-4A6) also reacted with cap-like structures localized at the periphery of the cytoplasm but not protruding into the spike-like cytoplasmic processes, suggesting that the cap structures formed a submembranous sheet.

In mitotic cells, all pNDCFs rearranged into filamentous structures that formed a perichromosomal basket. In interphase cells, the nuclear dots were located in the euchromatin, or if they were located in the heterochromatin, they were surrounded by a narrow rim of low DNA density chromatin, as demonstrated by double-staining Hoechst 33258. Double immunofluorescence staining with either rabbit anti-PML or biotinylated anti-EBNA-5 antibodies showed that pNDCF nuclear dots were different from the PML/EBNA-5/WHO-positive nuclear blobs.

The filamentous cytoplasmic staining seemed to be granular at high magnification, covering IF-like cytoplasmic bundles. Double immunofluorescence staining with five mAbs and biotinylated anti-vimentin mAb detected a close association between vimentin and the cytoplasmic pNDCFs in both IB4 cells and primary human fibroblasts (Fig. 3).

Interestingly, the cytoplasmic filaments frequently seemed to be connected to the nuclear dots by weak nuclear filamentous material. Dual-color optical sectioning was used to verify that these filament-like structures were located inside the nucleus, rather than being an out of focus optical projection of cytoplasmic filaments that closely followed the

<table>
<thead>
<tr>
<th>Morphological category</th>
<th>No. of clones</th>
<th>Staining pattern in IB4 cells</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>11</td>
<td>Speckled fluorescence staining restricted to the nucleus in 70–100% of the cells</td>
<td>pSN-Maj</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>Speckled fluorescence staining restricted to the nucleus in 1–70% of the cells</td>
<td>pSN-Min</td>
</tr>
<tr>
<td>C</td>
<td>7</td>
<td>Nuclear dots, nuclear filaments, cytoplasmic filaments, in mitotic cells rearranged into perichromosomal basket</td>
<td>pNDCF</td>
</tr>
<tr>
<td>D</td>
<td>12</td>
<td>Nuclear dots with other cytoplasmic and membrane structures</td>
<td>pNCM</td>
</tr>
<tr>
<td>E</td>
<td>6</td>
<td>Various cytoplasmic and membrane staining avoiding the nucleus</td>
<td>pCM</td>
</tr>
</tbody>
</table>
nuclear surface (Fig. 2). Examination of 16 nuclei indicated that the nuclear foci within the euchromatic areas, but not those in the heterochromatin regions, were connected to the filamentous structures.

Biochemical Analysis of pNDCFs. Immunoblotting of total cell lysates from IB4 or IARC171 LCLs showed that the anti-pNDCFs reacted with high Mr (>200,000) proteins that had slightly different Ms. They did not cross-react with vi-
Fig. 3. Colocalization of pNDCFs (yellow) with vimentin (red) in LCL IB4 (A–C) and human fibroblast (D–F). A and D, immunofluorescence staining with DM-10H2 and DM-6A2, respectively (FITC); B and E, vimentin detected by biotinylated anti-vimentin mAb (Texas Red). C and F, overlap of the staining patterns. DNA staining, blue.

Fig. 4. Western blot of the different pNDCFs from the total lysates of the very strongly positive IARC171 lymphoblastoid cells (A) and the almost negative Jioye M13 BL line (B) on an 8% polyacrylamide gel. B, absence of cross-reaction with vimentin in total cell lysate of IB4 lymphoblastoid cells on Western blot using a 7% polyacrylamide gel. The lysate was run from a single preparative well and probed with different antibodies using a multiwell device.
Table 2  pNDCF expression in different cell lines in vitro

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DM-1B5</th>
<th>DM-4A6</th>
<th>DM-5C6</th>
<th>DM-6A2</th>
<th>DM-10H2</th>
<th>Vimentin</th>
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<tr>
<td>K562&lt;sup&gt;e&lt;/sup&gt;</td>
<td>++</td>
<td>++</td>
<td>+/−</td>
<td>+</td>
<td>++</td>
<td>+</td>
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<tr>
<td>U937&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Saos-2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Human fibroblast&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
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<tr>
<td>RHEK-1&lt;sup&gt;p&lt;/sup&gt;</td>
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<td>+</td>
<td>+</td>
<td>++</td>
<td>+/−</td>
<td>+/−</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>MolT-4&lt;sup&gt;p&lt;/sup&gt;</td>
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<td>+/−</td>
<td>+/−</td>
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<tr>
<td>HL60&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
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<tr>
<td>HeLa&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>Lewis lung cc.&lt;sup&gt;i&lt;/sup&gt;</td>
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<td>+</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>DF W&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>+</td>
<td>+++</td>
<td>+++</td>
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<td>+</td>
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<tr>
<td>COS-1&lt;sup&gt;i&lt;/sup&gt;</td>
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<td>+</td>
</tr>
<tr>
<td>CV1&lt;sup&gt;i&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

* + to ++++, relative staining intensity; ND, not done because the anti-vimentin mAb is human-specific.
<sup>a</sup> Human erythroleukemia.
<sup>p</sup> Human promonocytic leukemia.
<sup>e</sup> Human osteosarcoma.
<sup>i</sup> Human embryo lung fibroblast, passage no. 18.
<sup>b</sup> SV40LT-immortalized human keratinocyte.
<sup>d</sup> Human breast carcinoma.
<sup>c</sup> Human cervical carcinoma.
<sup>o</sup> Human T-cell lymphoma.
<sup>f</sup> Human myelomonocytic leukemia.
<sup>q</sup> Exclusively submembrane caps.
<sup>j</sup> Exclusively nuclear staining.
<sup>g</sup> Human cervical carcinoma.
<sup>g</sup> Exclusively submembrane caps.
<sup>q</sup> Immortalized monkey kidney epithelial cells with SV40LT.
<sup>i</sup> Immortalized monkey kidney epithelial cells without SV40LT.

Vimentin by immunofluorescence staining or on the Western blot. Control cells, which were negative for pNDCFs by immunofluorescence, were also negative on Western blot (Fig. 4). Treatment of LCLs or human fibroblasts with cytoskeleton extraction buffer before fixation failed to eliminate the staining of any of the pNDCFs, indicating that these proteins are firmly associated with cytoskeletal and nuclear matrix elements (data not shown).

**Expression Pattern in Different Cell Lines.** The pNDCFs were found to be ubiquitously expressed in mouse, monkey,
Table 3  Expression of pNDCFs in the tissues of 17-day-old SCID mouse embryos

<table>
<thead>
<tr>
<th>Cell/tissue type</th>
<th>DM-1B5</th>
<th>DM-4A6</th>
<th>DM-5C6</th>
<th>DM-6A2</th>
<th>DM-10H2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intervertebrate disc</td>
<td>++ +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Gum epithelium</td>
<td>+ + +</td>
<td>-</td>
<td>-</td>
<td>+ + +</td>
<td>+ / -</td>
</tr>
<tr>
<td>Tongue epithelium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ + +</td>
<td>+ / -</td>
</tr>
<tr>
<td>Esophagus epithelium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ +</td>
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<tr>
<td>Foregut epithelium</td>
<td>+ + +</td>
<td>-</td>
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<td>+ +</td>
</tr>
<tr>
<td>Stomach epithelium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ / -</td>
<td>-</td>
</tr>
<tr>
<td>Small intestine epithelium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ b</td>
<td>-</td>
</tr>
<tr>
<td>Colon epithelium</td>
<td>-</td>
<td>-</td>
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<td>Pancreas</td>
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<td>+ + +</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Liver (hepatocytes)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ b</td>
<td>-</td>
</tr>
<tr>
<td>Liver (blood-forming islands)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ / -</td>
<td>+ +</td>
</tr>
<tr>
<td>Kidney</td>
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<td>-</td>
<td>-</td>
<td>+ / -</td>
<td>+ +</td>
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<td>+</td>
<td>+ +</td>
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<tr>
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<td>+ / - b</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+ +</td>
<td>-</td>
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<td>+ +</td>
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<td>+ + +</td>
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<tr>
<td>Intervertebral web</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ +</td>
</tr>
<tr>
<td>Edges of the eyelids</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ +</td>
</tr>
<tr>
<td>Vestibular lamina in the oral cavity</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ +</td>
<td>+ +</td>
</tr>
</tbody>
</table>

* Tissues that are always negative included bone, tendon, endothelial, mesothelial, adrenal gland, lung, tooth bud, and placenta tissue. – to + + +, relative staining intensity.

b Exclusively nuclear staining.

and human cell lines of different tissue origin, as summarized in Table 2. They were associated with cytoplasmic filaments and, to a variable extent, with nuclear foci. The distribution of the former resembled IFs. In fibroblasts and lymphoid cells, they colocalized with vimentin. IF-like staining was also detected in 20–30% of the cells of immortalized human keratinocytes cell line RHEK-1 by four out of five mAbs (DM-1B5, DM-4A6, DM-6A2, and DM-10H2), although less than 1% of these cells expressed vimentin. This suggests that certain pNDCFs are able to associate with IFs other than vimentin, presumably different keratin subtypes, in epithelial cells. DM-5C6 only reacted with the vimentin-positive minority. Its target protein showed a high degree of colocalization with vimentin.

Double staining with rhodamin-conjugated phalloidin, which detects polymerized actin, showed a different pattern. None of the anti-pNDCF antibodies reacted with the mitotic spindle. Treatment with Colcemid did not disrupt the pNDCF filaments.

Although there were similarities between the staining patterns obtained with the different antibodies, there were individual differences. DM-5C6 and DM-6A2 showed intense cytoplasmic filament staining. DM-10H2 preferentially stained filament-associated coarse granules, whereas DM-1B5 stained mainly the nuclei. DM-4A6 often detected submembranous sheets in addition to cytoplasmic filaments and nuclear dots.

**Differentiation-dependent Expression in Mouse Embryos.** All five anti-pNDCF reacted with mouse antigens. To study the expression pattern of pNDCFs in normal tissues, we used frozen sections from close-to-term (17 days) SCID mouse embryos. In contrast to the almost uniformly reactive cultured cell lines, the fetal tissues showed a highly distinctive staining pattern for individual pNDCFs. The results are summarized in Table 3. With few exceptions, the staining was cytoplasmic and associated with filament-like structures. The only tissue that was stained with all antibodies at this stage of development was the intervertebrate disc. All other cell types regularly expressed one or two pNDCFs at the same time. If two pNDCFs were present in the same cell, sometimes they were localized to different cellular compartments, one in the nucleus and the other in the cytoplasm. One antibody, DM-10H2, detected cells, among others, that were programmed to die by apoptosis at a later stage of development (e.g. cells in the interdigital web, cells between the unopen eyelids, or cells in the oral vestibular lamina).

**Expression Pattern in Resting, Mitogen-stimulated, and EBV-infected B Cells, LCLs, and BL Lines.** Resting human B cells expressed low antigen levels, stained as nuclear dots and cytoplasmic filaments. Activation by EBV or by anti-CD40 antibody and IL-4 (Fig. 5) induced a marked increase in the staining intensity of the nuclear blobs and the cytoplasmic filaments. LCLs showed intense staining with strong cytoplasmic filaments. In contrast, type I BL lines
were either completely negative or showed only nuclear blob staining without any cytoplasmic filaments. EBV-carrying BL lines that shifted to a more LCL-like phenotype preferentially expressed cytoplasmic filament-associated antigens. This expression was restricted to only a relatively small proportion of the cells and showed a good but not absolute correlation with the appearance of vimentin (Table 4). The submembrane cap structures, detected by the DM-4A6 mAb, were also strongly induced by EBV or mitogenic stimulation of B cells. The caps were also present in some of the type I BL lines.

Discussion
The nuclear matrix is believed to play an important role in DNA replication (19, 20) and in the regulation of transcription (21–23). Moreover, it provides a structural basis for nuclear compartmentalization by defining separate domains. The matrix is composed of nuclear lamina located at the nuclear periphery, an intranuclear meshwork of IF-like filaments with 23-nm axial repeats (24), different intranuclear bodies, and the fibrillar component of the nucleolus.

The molecular components of the nuclear matrix are relatively well known. The major constituents are different lamins (types A, B, and C) that provide most of the material for both the nuclear lamina and intranuclear filaments (25). On the other hand, the field of matrix-associated proteins remains largely unexplored, mainly due to the technical difficulties in studying protein-protein interactions in insoluble cellular remnants.

In this paper, we present five antigens (pNDCFs) that are apparently members of the same protein family. Alternatively, the pNDCFs might be differentially spliced or posttranslationally modified products of the same gene. These questions can be resolved only by cloning the genes of the different pNDCFs. The pNDCFs are strongly associated with the nucleoskeleton and cytoskeleton. The distribution of these proteins differs from that of known nuclear and cytoplasmic proteins. Searching the MEDLINE from 1966 to 1996, we could not find data in the literature on other proteins that associate with IFs in the cytoplasm and can form distinct nuclear bodies and associate with IFs during cell division. No primate or rodent proteins in the Swissprot protein database with a Mr, higher than 170,000 match this pattern of distribution.

There are literature reports about several high molecular weight cytoskeleton-associated proteins, some of which bind to IFs. Most of these proteins avoid the nucleus, however.

An IgM antibody that reacted with both desmin and vimentin was recently reported to recognize distinct nuclear
foci as well (26). It is possible that this antibody can react with the pNDCFs. Our antibodies do not cross-react with vimentin by immunostaining and Western blot.

The subcellular localization of the pNDCFs is puzzling, because they can apparently form a morphological continuum between the cell membrane and defined nuclear domains. It is conceivable that pNDCFs may be involved in signal transduction and/or molecular transport between the membrane and specific regions of the chromatin. It is believed that vimentin is directly anchored to the nuclear lamina due to its binding to lamin B (27) and the peripheral cytoskeletal mesh by ankyrin (28). The pNDCFs would extend this molecular continuum by their entry into the nucleus and by their arrival to distinct nuclear targets. Their filamentous appearance may result from their association with existing nucleoskeletal filaments.

High molecular weight IF-associated proteins are used as histological markers in tumor diagnosis. This can be exemplified by the 160-kDa restin that is highly expressed in cultured lymphoid cells and tends to accumulate in Reed-Sternberg cells in Hodgkin’s disease (29) as well as in anaplastic large cell lymphomas, but not in normal lymphoid tissues (30). Restin stimulated cell proliferation upon expression from a transfected vector (31).

Very high molecular weight proteins like IFAPA-400 and IFAP-300 were found to be associated with IFs in developing neurons, glial cells, and muscle during specific phases of embryonic development. It was suggested that they play a role in the regulation of cell differentiation (32–34). IFAP-300 is expressed in human astrocytomas but not in normal or reactive astrocytes, providing a diagnostic tool for the recognition of transformed cells (35). IFAP-300 is associated with vimentin filaments in interphase cells but dissociates from them during mitosis (36). The most ubiquitous expression of pNDCFs in in vitro cell lines of transformed origin in contrast to the very restricted expression pattern in normal tissues hints that some of them could be used as tumor progression markers in certain malignancies.

EBV activation of human B cells is believed to mimic physiological activation by the cognate antigen (5, 37). Here we showed that pNDCFs are induced in parallel with B-cell activation by EBV or IL-4 and anti-CD40. EBV induces major rearrangements in the organization of the cytoskeleton (38). EBV transformation of B cells is accompanied by vimentin induction. In EBV-negative BL lines, transfected latent membrane protein 1 (39) or EBNA-4 (40) could induce vimentin expression.

The absence or exclusively nuclear localization of pNDCFs in BL lines can be partly explained by the characteristic absence or low level expression of vimentin (41). The in vitro shift of EBV carrying BL lines toward a more LCL-like phenotype is concomitant with an increased expression of vimentin. Some of the type III BL lines express cytoplasmic filament-associated pNDCFs, especially proteins detected by DM-5C6, DM-6A2, and DM-10H2. Because all LCLs express filament-associated pNDCFs, they should be included in the molecular definition of the type III phenotype.

Materials and Methods

Cells and Cell Culture. All cell lines were cultured at 37°C in Iscove’s medium containing 10% FCS and 50 μg/ml gentamicin. Absence of mycoplasma contamination was monitored by periodic staining with Hoechst 33258. Isolation of human CD19-positive B cells, mitogenic stimulation by anti-CD40 antibodies and IL-4, and infection with B95-8 strain of EBV were carried out as described previously (12, 18).

Preparation of Antigen, Immunization, and Generation of Hybridomas. A total of 5 × 107 logarithmically growing human LCL cells (IB) were washed twice with PBS and fixed with methanol-acetone (1:1). The cells were rehydrated with PBS and, after two additional washes, sonicated at 0°C in a volume of 2 ml with four bursts of treatment at energy level 22 in a Soniprep 150 MSQ sonicator. The lysate contained only cell fragments smaller than 0.5 μm. This lysate was incubated with Dynabeads loaded with sheep anti-mouse IgG and saturated by the anti-EBNA-5 mouse mAb JF186 (42).

The beads were washed three times with PBS and injected into the spleen of six BALB/c mice as described previously (43). The injection was repeated 12 days later, and the mouse that showed the highest antinuclear immunostaining compared to the preimmune serum was used for hybridoma fusion. The isolated spleen cells were fused with Sp2/0 cells by the stirring method as described by Harlow and Lane (44). The clones were plated on a syngeneic peritoneal macrophage feeder layer in Iscove’s medium containing 20% FCS and hypoxanthine-aminopterin-thymidine-selective medium.

The clones from 10 plates were tested for immunoglobulin production by a nitrocellulose filter assay. The best 100 clones were tested further for immunostaining on IBA4.

Production of mAbs. In the first phase of the experiments, the cell-free buffered supernatants of the clones were used as staining reagents. Hybridomas producing the desired mAbs were also injected into the i.p. cavity of Pristane oil-primed SCID mice to raise high-titer ascites, free of contaminating host immunoglobulins.

Immunofluorescence Staining and Image Analysis. The cells were regularly fixed with methanol-acetone (1:1) for at least 10 min and rehydrated in PBS. The supernatant antibodies were diluted in blocking buffer (2% BSA, 0.2% Tween 20, 10% glycerol, and 0.05% NaN3 in PBS) in the proportion of 1:1 and 1:50–1000, respectively, depending on the individual antibodies. The first antibody was incubated with the cells for 30 min at room temperature followed by three-2-min washes. The secondary antibody, regularly FITC-conjugated rabbit anti-mouse IgG (Dakopatts) was diluted in blocking buffer (1:20). Biotinylated of the vimentin antibody and the double fluorescence staining were done as described previously (15). Extraction of cells with nonionic detergent containing buffer was carried out as described previously (45). The slides were mounted with 70% glycerol and 2.5% DABCO (Sigma; pH 8.5) in PBS. The images were generated using a Leitz DM RB microscope equipped with Leica PL Fluotar ×100, ×40, and PL APO Ph oil immersion objectives. Leica L4, Tx, and A composite filter cubes were used for the FITC, Texas Red, and Hoechst 33258 fluorescence, respectively. The pictures were captured with a Hamamatsu dual-mode cooled charge-coupled device camera (C4880), recorded, and analyzed on a Pentium PC (133 Mhz, 32 Mb RAM) computer equipped with an AFG VISIONplus-AT frame grabber board using the Hipic3.2.0 (Hamamatsu), Image-Pro Plus (Media Cybernetics), and Adobe Photoshop image-capturing and processing software.

The raw monocolor images were captured as black-and-white 12-bit ITKS-compatible IMAGING files and were converted into 8-bit TIFF files. The color composite images were generated by merging the individual 8-bit files into the 24-bit RGB TIFF file, assigning the FITC fluorescence as the green channel, Texas Red fluorescence as the red channel, and Hoechst fluorescence or calculated red/green overlap as the blue channel. The overlap calculation and image-merging program routines were developed by us, using the Image-Pro Plus macro functions.

Expression Studies in Mouse Fetuses. The 8-μm-thick parasagittal frozen sections were cut from 17-day-old SCID mouse embryos. The sections were fixed in methanol-acetone (1:1), stained, and analyzed as described previously (46).

Western Blotting. Detection of membrane-blotted proteins was carried out as described previously (47), with the modification that chemilu-
minescence (enhanced chemiluminescence kit; Bio-Rad) was used to detect the peroxidase-conjugated sheep anti-mouse IgG.

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