Two short autoepitopes on the nuclear dot antigen are similar to epitopes encoded by the Epstein–Barr virus

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ABSTRACT To understand the relationship between antibodies present in patients with anti-nuclear dot (ND) autoimmune disease and the proteins they recognize, epitopes that react with the autoantibodies were mapped. A panel of fusion proteins containing different portions of the ND protein were overproduced in Escherichia coli. Immunoblot analysis with anti-ND antibodies revealed that most (10 of 12) sera recognize two major autoepitopes that are each a maximum of 8 amino acids long. The other two sera recognize one of the two epitopes. In addition to the short linear autoepitopes, a conformational epitope appears to be present on the ND antigen. Each of the two linear epitope sequences shares sequence similarities with those of several viral proteins found in the databases. Furthermore, two fusion proteins containing short Epstein–Barr virus (EBV) protein sequences that are similar to the ND epitopes were recognized by the human autoimmune sera, indicating that the autoepitopes are present in EBV protein sequences. Our results are consistent with the hypothesis that ND autoimmune disease might be associated with EBV infections.

Anti-nuclear antibodies are found in a variety of different autoimmune disease patients. In many cases, the reactivity of the antibody produced often correlates with the particular disease (e.g., ref. 1). Despite this fact, the etiology of both the disease and antibodies is unknown. Furthermore, since many autoantigens reside in the nucleus and are, therefore, presumably inaccessible to autoantibodies, it is also unclear to what extent the antibodies contribute to the pathogenesis, especially in the initial stages of the autoimmune disease.

Several models have been proposed to account for the etiology and pathogenesis of autoimmune disease. One of these, the molecular mimicry model (2), states that an infecting agent such as a bacteria or virus might be involved in the onset or pathology of the disease. Antibodies produced against the infecting agent might cross-react with cellular antigens and, potentially, contribute to the disease state.

Recently, efforts have been directed toward mapping epitopes recognized by antibodies isolated from patients with different types of autoimmune diseases. Thus far, epitopes have been mapped for a variety of different autoantigens, and some of these are similar to viral sequences (3–6). For example, autoepitopes have been mapped on the α subunit of the acetylcholine receptor that is associated with myasthenia gravis (3) and on the diffuse systemic sclerosis antigen DNA topoisomerase I (4). The α subunit of acetylcholine receptor exhibits immunological cross-reactivity with a homologous domain on herpes simplex virus glycoprotein D (3), while the antigenic 11-amino acid region of DNA topoisomerase I contains a 6-amino acid sequence that is identical to a sequence present in the group-specific antigen (p30α) of some mammalian retroviruses (4). However, the usefulness of these studies is limited because in most instances mapping was done at low resolution and only a single epitope was identified. Thus, the correlation between the mapped epitope and viral sequence is usually weak.

We (7) and others (8, 9) have been studying anti-nuclear dot (ND) autoimmune disease, in which the autoantibodies brightly stain 4–12 dots in the nucleus (7–9). Anti-ND antibodies are commonly found in patients with primary biliary cirrhosis, but they are also found in patients with other types of autoimmune disease, such as systemic lupus erythematosus and rheumatoid arthritis. The antigen recognized by anti-ND antibodies is 480 amino acids long and migrates as a 72-kDa polypeptide by SDS/PAGE (7). The gene encoding this protein has been cloned and sequenced (7, 9).

In this study, we used the cloned gene to map epitopes recognized by the anti-ND antibodies. Two major epitopes were mapped at high resolution and found to be similar to sequences present on several viral proteins. We show that two Epstein–Barr virus (EBV) sequences are recognized by ND autoimmune sera, raising the possibility that EBV could be involved in the production of these human autoimmune sera and in the etiology of its associated diseases.

MATERIALS AND METHODS

Sera, Immunofluorescence, and Immunoblot Analysis. Rabbit anti-ND polyclonal antibodies and seven human anti-ND autoimmune sera have been described (9). Four other human sera were provided by M. Semenov (Yale University) and a fifth serum was provided by J. Evans and J. Craft (Yale University). Indirect immunofluorescence was conducted by using cells that were grown on coverslips and fixed with 1% formaldehyde in PBS for 10 min at room temperature as described by Yang et al. (10). Immunoblot analysis was performed as described (7).

Construction of Fusion Proteins for Epitope Mapping. TrpE fusion proteins (11) and glutathione S-transferase (GST) fusion proteins (12) were used in this study. The pATH–ND–trpE fusion is described in Xie et al. (7). Truncated TrpE–ND fusion plasmids were made by cleaving the pATH–ND plasmid at Pst I (nt 285–468), Xba I (nt 285–435), and BamHI (nt 285–333). The ends were made blunt by using the large fragment of DNA polymerase I, and then the plasmids were recircularized with DNA ligase.

To produce GST–ND fusion proteins, different fragments were synthesized by PCR (13) with oligonucleotide primers and full-length ND as template DNA. The primers contained either an EcoRI or BamHI site near the end of the upstream primer and an EcoRI site near the end of the downstream primer. The PCR products were cleaved with EcoRI and/or BamHI and cloned into pGEX-2T. Constructs containing residues 372–383 (EII.1), 372–379 (EII.2), 374–381 (EII.3), and 376–383 (EII.4) were prepared by oligonucleotide synthesis as described below. Proper preparation of all constructs used in this study was verified by DNA sequence analysis.

Abbreviations: ND, nuclear dot; EBV, Epstein–Barr virus; GST, glutathione S-transferase; EI, epitope I; E2, epitope II.
Synthesis of Viral Sequences. Oligonucleotides were synthesized for all viral sequences and the EII.1, EII.2, EII.3, and EII.4 ND sequences (54 nt long for the viral sequences and 54–72 nt for the ND sequences). The viral sequences (24 nt) or ND sequences (24–36 nt) were flanked on their 5’ side by the sequence GGTAACCGGGGATTC (which contains a BamHI site) and on the 3’ side by GGAATTCGATGCAA (which encodes an EcoRI site), respectively. The oligonucleotides were then made double-stranded by using primers identical to the 5’ flanking sequences and complementary to the 3’ flanking sequences mentioned above (TTGCATGCGAATTC and GGTAACCGGGGATTC) and PCR.

Antibody Depletion Assay. Autoimmune serum 312 diluted 1:3000 in PBS (~100 μl) was incubated with ~100 pmol (2–8 μg) of each GST fusion protein containing (i) the epitope sequences, (ii) the C-terminal fragment of ND protein containing the two major epitopes, (iii) the full-length ND protein, or (iv) ~2 μg of full-length ND fusion protein (in 20 μl of water) heated to 100°C for 15 min. The fusion protein is present in at least a 50-fold excess relative to the antibody. The GST protein without ND sequences was used as a control. Antibodies were incubated in the presence of the GST proteins on ice for 1 hr. An excess amount of glutathione-agarose beads (Sigma) was added, and the solution was centrifuged at 10,000 × g for 10 min to remove the GST fusion protein and absorbed antibodies. The supernatant was used for indirect immunofluorescence on HeLa cells.

RESULTS

Anti-ND Autoimmune Sera. Twelve anti-ND autoimmune sera from autoimmune disease patients (Table I) were used for this study. Each serum was found to recognize the same antigen as shown by the following criteria: (i) Each serum stains 4–12 dots per nucleus in HeLa cells (see Fig. 4, for the immunofluorescence pattern for serum 312), and double immunofluorescence with affinity-purified rabbit antibodies generated to the cloned ND protein (7) revealed that for each human serum the human antibodies and rabbit antibodies co-localize. Thus, these sera recognize the same nuclear domains.

Table I. Profile of ND autoimmune sera

<table>
<thead>
<tr>
<th>Group of serum</th>
<th>Immunoblot (72 kDa)</th>
<th>ND epitopes</th>
<th>Disease or symptom</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ND rabbit serum</td>
<td>+ + +</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Human serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>312</td>
<td>+ + +</td>
<td>SLE</td>
<td></td>
</tr>
<tr>
<td>397</td>
<td>+ + +</td>
<td>PBC</td>
<td></td>
</tr>
<tr>
<td>258</td>
<td>+ + +</td>
<td>PBC</td>
<td></td>
</tr>
<tr>
<td>810</td>
<td>+ + +</td>
<td>PBC</td>
<td></td>
</tr>
<tr>
<td>699</td>
<td>+ + +</td>
<td>ANA</td>
<td></td>
</tr>
<tr>
<td>797</td>
<td>+ + +</td>
<td>SLE, MCTD</td>
<td></td>
</tr>
<tr>
<td>578</td>
<td>+ + +</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>265</td>
<td>+ + +</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>893</td>
<td>+ + +</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>K965</td>
<td>NT + +</td>
<td>RA</td>
<td></td>
</tr>
<tr>
<td>Group C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K871</td>
<td>NT +</td>
<td>SLE</td>
<td></td>
</tr>
<tr>
<td>P208</td>
<td>NT +</td>
<td>SLE</td>
<td></td>
</tr>
</tbody>
</table>

Patient profiles and reactivities of anti-ND sera. The 12 human sera were divided into groups A, B, and C by their recognition pattern of the ND autoepitopes. All sera were ND-positive (4–10 dots per nucleus) by indirect immunofluorescence. SLE, systemic lupus erythematosus; PBC, primary biliary cirrhosis; ANA, antinuclear antibody; MCTD, mixed connective tissue disease; RA, rheumatoid arthritis; NT, not tested; NA, not applicable.

Fig. 1. Linear epitope mapping strategy for ND protein and reactivities of different TrpE- and GST–ND fusion proteins. (A) Low-resolution analysis of the entire ND protein. (B) High-resolution analysis of the reactive C-terminal region. Immunoblots of fusion proteins such as those shown in Figs. 2 and 3 were probed with affinity-purified rabbit polyclonal serum (aND). The 12 human autoimmune sera fell into three groups (groups A, B, and C; see Table 1). Numbers indicate the amino acid residues of the ND protein that were fused to GST. + and − indicate positive and negative immunoblot reactions, respectively. ORF, open reading frame.
Thus, the region of the ND protein that is reactive by immunoblot analysis resides in the C-terminal portion of the protein.

**Two Short Autoepitopes Are Recognized by Most of the Autoimmune Sera.** To map the epitope(s) more precisely within the C-terminal region, a second stage of epitope mapping was carried out (Fig. 1B). Oligonucleotide primers corresponding to various positions along both strands of the ND cDNA were used to amplify different ND coding sequences that were then cloned into a GST fusion vector. In some cases, specific oligonucleotides were synthesized and cloned into the same vector. A total of 24 fusion proteins containing different portions of ND coding sequences were overproduced and probed with each of the anti-ND autoimmune sera (Fig. 1B). The fusion proteins were also probed with the rabbit anti-ND serum (both affinity-purified and crude serum) and with rabbit preimmune serum. Three sample blots probed with human sera 312 and 893 and the affinity-purified rabbit serum are shown in Fig. 2.

The 12 anti-ND autoimmune sera fell into three categories. The majority (8 of 12) of sera (group A) recognized two major epitopes, called epitope I (EI) and epitope II (EII), respectively. Each reactive region was mapped to 8 amino acids (residues 342–349 for EI; residues 376–383 for EII). GST–ND fusion proteins containing only these 8 amino acids react strongly with the autoimmune sera (EI and EII.4 in Figs. 1 and 3). Similar-sized or larger fragments from other portions of the ND C-terminal region did not react with these sera (e.g., see Fig. 2, constructs 400–435 and 400–480 for serum 312). For EI, the minimum size shorter than 8 amino acids was not determined. For EII, coding segments that lack 2 amino acids from the N-terminal sequences (construct 378–386 in Fig. 1; immunoblot data not shown) or the C-terminal end (EII.3 in Fig. 1 and EII.3 in Fig. 3) were not recognized by the autoimmune sera. Thus, this epitope is probably 6–8 residues long.

**FIG. 2.** Sample immunoblots of 13 GST–ND fusion proteins. The top panel depicts a Coomassie blue-stained gel containing ~0.2 μg of the GST fusion proteins indicated. The numbers above the gel denote the residues in the ND protein that are fused to GST. The bottom three panels show immunoblot results in which similar gels containing 1/400th the amount of protein shown in a were probed with affinity-purified rabbit polyclonal ND serum (a) and two human anti-ND autoimmune sera, serum 312 (b) and serum 893 (c). None of the sera reacts with the GST protein without ND sequences (GST).

**FIG. 3.** Immunoblot of ND autoepitope sequences and viral ND epitope homologous sequences. The top panel shows a Coomassie blue-stained gel with ~0.2 μg of GST fusion protein. (a–c) Immunoblots containing 1/400th of the same samples were probed with affinity-purified rabbit serum (a), human serum 312 (b), and human serum 397 (c) (see Figs. 1 and 4 for the fusion proteins).

Of the remaining four sera, two, sera 893 and 995 in group B (Fig. 1), and group A sera had similar specificity. These sera recognized the two major epitopes and also weakly recognized a third segment, located very close to the C terminus. Constructs containing residues 400–480 and 400–436 reacted with these sera (see Figs. 2c and 1B), indicating that the epitope(s) resides in the region of residues 400–480. The remaining two sera recognized only EII (group C sera in Fig. 1). Thus, in summary, all 12 ND sera recognize EII, 10 of 12 recognize EI, and 2 sera weakly recognize a third region in addition to EI and EII.

The rabbit polyclonal serum (both crude and affinity-purified) recognized nearly all of the fusion proteins, including EI and EII, indicating that this serum recognizes many portions of the ND protein (Figs. 1–3). Only five fusion proteins that contained very short portions of the ND sequence were not recognized. The rabbit preimmune serum and one human control autoimmune serum [serum 578 (7)] that does not recognize NDs did not react with any of the fusion proteins (data not shown).

**A Conformational Epitope(s) Is Also Present on the ND Protein.** The procedures described above use denaturing gels and immunoblot analysis to identify autoepitopes. This method identifies short and/or "linear" epitopes but may miss "conformational" epitopes that are present on native forms of the protein. To determine whether anti-ND antibodies might also recognize conformational epitopes, indirect immunofluorescence and antibody depletion assays were used. An anti-ND serum (312; group A serum) was preabsorbed to a 50-fold or greater excess of GST fusion proteins containing either full-length ND sequences or EI and EII coding sequences. The reactive antibodies were removed by using glutathione-agarose beads, and the remaining antibodies were used to stain HeLa cells by indirect immunofluorescence to determine whether any anti-ND antibodies remained.

As shown in Fig. 4B, immunodepletion with a GST protein lacking ND sequences did not significantly remove the anti-ND antibodies, as determined by indirect immunofluorescence. In contrast, incubation with the full-length ND–GST
Comparison of two GST–ND fusion proteins: the GST–ND fusion protein (residues 331–436) (EI and EII) (C), GST–ND fusion protein (residues 1–480, full length) (E), and denatured GST–ND (full length) (F). The bound serum was removed from the solution, and the unbound serum was used to stain HeLa cells.

Fusion protein completely removed the ND staining (Fig. 4E), indicating that this serum only recognizes epitopes on the cloned ND protein. Immunodepletion of the ND serum with an excess of a GST fusion protein that contains either the C-terminal domain of the ND protein (residues 331–436, see Figs. 1 and 4D) or a mixture of two GST proteins (one fused to EI and the other fused EII; Fig. 4C) greatly reduced but did not eliminate the immunofluorescence signal. The staining was similar to that observed when denatured full-length ND–GST fusion protein was used for the immunodepletion experiments (Fig. 4F). The presence of some staining indicates that a conformational epitope(s) probably exist on the ND protein.

Two other conclusions can be drawn from these results. (i) Since the mixture of fusion proteins containing both epitopes appears to diminish the immunofluorescence signal to the same extent as the entire C terminus (see Fig. 4 C and D), it is unlikely that other nonconformational epitopes exist in the C-terminal region for serum 312. (ii) Since most of the signal can be removed from the serum by using the mixture of EI– and EII–GST fusion proteins, EI and EII appear to be the major epitopes present in the ND autoimmune sera.

**ND Autoimmune Sera Can Recognize Epitopes on EBV Proteins.** As mentioned above, viruses containing epitopes that cross-react with cellular antigens have been suggested to be involved in autoimmune diseases. Comparison of the ND epitope sequences to others in the databases by BLAST analysis (14) revealed four viral sequences among the top 15 scores that were similar to EI (EVFISAPPR) and three viral sequences among the top 12 scores that were most similar to EII (PQIVPPEPM). The closely related viral sequences plus several other viral sequences are presented in Table 2. The sequences are from several different viruses (EBV, cowpox, mumps, visna, lymphotropic polyoma, and vesicular stomatitis NJ). Sequence similarity searches using randomized versions of the EI and EII amino acids did not identify any of these sequences.

**Table 2. Sequences of viral proteins homologous to ND EI and EII and their reactivities with rabbit and human sera**

<table>
<thead>
<tr>
<th>GenBank name</th>
<th>Epitope name</th>
<th>Sequence</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND EI</td>
<td>EI</td>
<td>EVFISAPPR</td>
<td>+</td>
</tr>
<tr>
<td>UL37_EBV</td>
<td>EI–V1</td>
<td>P----P----</td>
<td>+</td>
</tr>
<tr>
<td>MUMLP_1</td>
<td>EI–V2</td>
<td>DI----VS----</td>
<td>+</td>
</tr>
<tr>
<td>HI38_COWPX</td>
<td>EI–V3</td>
<td>N----P----</td>
<td>–</td>
</tr>
<tr>
<td>EBV_55</td>
<td>EI–V4</td>
<td>D---RT----</td>
<td>–</td>
</tr>
<tr>
<td>ND EII</td>
<td>EII4</td>
<td>PQIVPPEPM</td>
<td>+</td>
</tr>
<tr>
<td>EBN4_EBV</td>
<td>EII–V1</td>
<td>--VL----T--</td>
<td>–</td>
</tr>
<tr>
<td>KITH_EBV</td>
<td>EII–V2</td>
<td>TTV------</td>
<td>+</td>
</tr>
<tr>
<td>ENV_VILV</td>
<td>EII–V3</td>
<td>AE--I--A--</td>
<td>–</td>
</tr>
<tr>
<td>RRPL_VSVV</td>
<td>EII–V4</td>
<td>--FI--D--G</td>
<td>–</td>
</tr>
<tr>
<td>COAI_POVLY</td>
<td>EII–V5</td>
<td>----QQQ----</td>
<td>–</td>
</tr>
</tbody>
</table>

Sequences are listed in order of their relative degree of sequence similarity, as determined by using the BLAST program. Identical residues are indicated by a dash. For EI and EII, the EBV amino acids that reacted with the sera were the first and third best scores, respectively, that were identified in similarity searches with all proteins in the databases.

To test whether any of these sequences might be recognized by anti-ND autoimmune sera, oligonucleotides encoding these nine peptides were cloned into the GST fusion vectors, and fusion proteins were overproduced and probed with serum 312 (Fig. 3). Two fusion proteins, one containing viral epitopes EI–V1, which is similar in sequence to EI, and another containing EII–V2, which is similar to EII, reacted strongly with the ND autoimmune sera. Both peptides are derived from EBV proteins: EI–V1 resides in the BOLF1 protein, a poorly characterized protein; EII–V2 corresponds to a region in the thymidine kinase protein. The other viral protein sequences listed in Fig. 4 were not recognized by the ND serum, even though some are only 3 amino acids different from the reactive ND sequences. Thus, the two epitopes mapped in the ND protein have cognates encoded in EBV.

**DISCUSSION**

In this study, fusion proteins and immunoblot analysis were used to demonstrate that the ND autoantigen contains two major autoactive epitopes that are recognized by most of the human autoimmune sera tested. The linear autoepitopes were mapped to within a resolution of 8 amino acids and lie 27 amino acids apart from one another. Although two human sera (group B) recognize an epitope(s) other than the EI and EII, this additional epitope(s) is likely to be minor, as judged by the intensity of signals on immunoblots.

In addition to the linear epitopes, the antibody depletion assays demonstrated that the ND protein also has a weak conformational epitope(s). This conformational epitope(s) might reside in a portion of the ND protein that must be folded properly or in two or more discontinuous segments of ND protein that are brought together in the native form of the protein. It is possible that other conformational epitopes exist in the ND protein but might not be recognized in our indirect immunofluorescence experiments because of sample fixation conditions. However, the mild fixation conditions that were used are expected to minimize this concern. Regardless of whether other epitopes exist or not, the assay used here is a useful approach for detecting the presence of conformational epitopes.

The two ND epitopes were found to have cognates encoded by EBV that can be recognized by the sera. The EI sequence corresponds to an ~1200-codon EBV open reading frame called BOLF1 (15). Expression of a BOLF1 protein has not yet been reported and its function is not yet known. The EII sequence on EBV is present in a thymidine kinase, which is expressed during infection (16). Sequences similar to EI and
EIg on other viruses did not react with the anti-ND sera. The fact that both epitopes recognized by anti-ND antibodies are encoded by EBV substantially increases the likelihood that the cross-reaction is not simply serendipitous and that EBV may play a role in ND autoimmune disease. If we assume that both epitopes are each the equivalent of 5 amino acids of non-degenerate sequence and that all 20 amino acids are present at equal frequencies, then the probability of a 10-amino acid sequence residing in a specific set of proteins (e.g., viral proteins) is in 20^10 or 1 x 10^-13. For comparison, the human genome has ~100,000 genes encoding proteins with an average size of 450 amino acids; this corresponds to 4.5 x 10^7 short linear continuous epitopes. Thus, very few organisms, particularly viruses, are likely to have both epitopes by chance.

Our results are consistent with the possibility that EBV is involved, either by itself or in conjunction with other viruses or factors, in ND autoimmune disease. EBV is a human herpes virus that is endemic in all human populations. Most people are infected with the virus in early childhood and then carry the virus for life. The fact that EBV is a widely distributed virus makes it a suitable candidate for the global occurrence of patients with this type of autoimmune disease (17).

There are several clinical observations that indicate that EBV can contribute to autoimmune disease. (i) Autoantibodies have been detected in patients with viral infections, including patients infected with EBV (18, 19), mumps or measles virus (20, 21), chickenpox virus (22), and human immunodeficiency virus (23). (ii) Chronic infection of goats with caprine arthritis-encephalitis virus is associated with rheumatoid arthritis-like joint lesions (24). Furthermore, the administration of virus vaccines has been occasionally associated with the occurrence of autoimmune diseases (25).

Rather than directly participating in the cause of the disease, it is also possible that EBV might play an indirect role in the production of anti-ND antibodies. Patients previously infected with EBV might be stimulated for the production of the anti-ND antibodies either through a subsequent event that leads to the onset of or a general nonspecific enhancement of autoantibody production. Given the high specificity of anti-ND antibodies for reaction with just a few major epitopes, we favor the hypothesis of a direct association of EBV with anti-ND disease. Anti-ND antibodies are not common among autoimmune diseases distinct from primary biliary cirrhosis (7) or in sera of normal individuals (M.S., unpublished data), consistent with a more direct correlation between these antibodies and a particular type of autoimmune disease.

Several previous studies (26–29) have implicated EBV in other types of autoimmune diseases. Most rheumatoid arthritis patients (70–90%) have antibodies directed against the rheumatoid arthritis precipitant, which is an antigen extracted from EBV-infected cells, and >90% of the patients have antibodies to the rheumatoid arthritis nuclear antigen (26). Some patients with acute polyarthritis were also found to have EBV (27–29). Thus, in addition to ND-associated diseases, EBV may be involved directly or indirectly in several types of autoimmune disease.

Since two epitopes were identified in this study and mapped to high resolution, it may be possible to develop a simple, inexpensive, and specific diagnostic test for anti-ND patients by using peptides containing the epitope sequences. Approximately 30% of primary biliary cirrhosis patients have anti-ND antibodies (7); hence an anti-epitope diagnostic test would be useful for detecting this particular subclass of individuals. The identification of autoepitopes is also potentially useful for therapeutic purposes.

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