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Mimicry Between the Hepatitis B Virus DNA Polymerase and the Antigenic Targets of Nuclear and Smooth Muscle Antibodies in Chronic Hepatitis B Virus Infection

Giorgia Mieli-Vergani, † and Diego Vergani 2 *

Autoantibodies to nuclear and smooth muscle are common in hepatitis B virus (HBV) infection. To understand their origin, we scanned protein databases and found that HBV-DNA polymerase (HBV-pol) shares 7–9 amino acid sequences with nuclear (MHC II trans-activator, nuclear pore core protein, nuclear mitotic apparatus, and polymyositis sclerosis Ag) and smooth muscle proteins (caldesmon and myosin). Twenty-mer peptides with relevant homologues and an irrelevant control peptide were constructed and ELISAs were established. Sixty-five children with chronic HBV infection, 104 patients with other chronic liver diseases (CLD), 36 patients with extrahepatic autoimmune diseases, and 24 healthy controls were investigated. Double reactivity to HBV-pol peptides and corresponding self homologues was observed in 40% of HBV-positive patients as compared with four (4%) with other chronic liver diseases, two (6%) with extrahepatic autoimmune diseases, and in none of the healthy controls (p < 0.001 for all). Double reactivity to myosin or caldesmon or their HBV-pol homologues was associated with anti-smooth muscle Ab positivity by immunofluorescence (p < 0.05 for both). HBV-positive sera double reactive for myosin or caldesmon and their homologous HBV-pol peptides also reacted with the native proteins on immunoblot. Fifty to ninety percent Ab inhibition to individual HBV-pol and HBV-pol<sub>49-118</sub> peptides was noted by preincubation with individual HBV-pol/self homologue peptide and native proteins, respectively, but not with control peptide. Our results show that cross-reactive immunity targeting homologous sequences of viral and self proteins may partly account for autoantibody production in HBV infection.


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We have recently demonstrated that anti-nuclear Abs (ANA) and anti-smooth muscle Abs (SMA) are part of the natural course of chronic hepatitis B virus (HBV) infection (1). Studying the presence and fluctuation of tissue autoantibodies in a cohort of patients undergoing an IFN-α controlled trial (2), we noted that the same proportion of treated and untreated patients were seropositive for ANA and SMA over the 4-yr observation period of the study. Moreover, positivity for ANA and SMA was not influenced by IFN-α in the treated group.

Since the production of non-organ-specific autoantibodies is a recognized consequence of viral infection and appears to be part of a virus-induced immune dysfunction, we investigated the possibility that these autoantibodies could be produced by an inappropriate host immune response to viral Ags. Thus, a host Ab response initially directed to HBV Ags could target structurally similar host components and lead to autoimmunity, a concept known as “molecular mimicry.” To address this hypothesis experimentally, we interrogated protein databases in search of sequence similarities between HBV proteins and putative antigenic targets of ANA and SMA. The HBV DNA polymerase harbored regions with the greatest homology to host Ags. We selected six nuclear and smooth muscle Ags with the highest local sequence similarity to HBV DNA polymerase for experimental investigation. Peptides encompassing these sequences were constructed and tested as targets of immune and cross-reactive autoimmune responses.

Materials and Methods

Patients

Sixty-five children (median age, 8 (2–16) yr; 39 boys) with HBsAg-positive (microparticle enzyme immunoassay (MIEA), Abbott, Chicago, IL) chronic hepatitis were studied. At the time of testing, 42 were HBV-DNA (dot blot assay, Abbott) and HBeAg (MIEA, Abbott) positive; four were HBV-DNA negative, HBeAg positive; 15 were HBeAg negative, anti-HBe positive; and four were positive only for HBsAg. All patients were negative for Abs to hepatitis C (United Biomedical, Hauppauge, NY), hepatitis D (Sorin Biomedica, Saluggia, Italy), and HIV (Access Immunoassay System, Sanofi Pasteur). Histological diagnosis (3) was chronic hepatitis with mild or moderate activity in 36 (55%) patients, chronic hepatitis with minimal activity in 15 (23%), nonspecific reactive hepatitis in seven (11%), and normal liver histology in four (6%). Three patients had no liver biopsy. Of the 65 patients, 44 received IFN-α therapy at 5 mega units/m²/dose thrice weekly either as lymphoblastoid IFN-α for 3 mo (n = 21) or recombinant IFN-α for 6 mo (n = 23). Twenty-one patients received no treatment.

The autoantibody profile of the majority of these patients has previously been reported (1). ANA, SMA, liver kidney microsomal type 1 (LKM1), and mitochondrial (AMA) autoantibodies were tested at a screening dilution of 1/10 in PBS using frozen rat liver, kidney, and stomach as substrate. A polyclonal anti-human Ig (IgG, IgA, IgM; Dako, Copenhagen, Denmark).
was used as a second reagent at a dilution of 1:20 in PBS. All positive sera were double diluted to extinction.

Presence of autoantibodies was tested on 365 serum samples (median of 6 per patient) collected over a period of 1 to 6 yr (median 3 yr). ANA and SMA at a titer ≥1/10 were detected on at least two occasions in 43 patients (32 treated patients and 11 untreated) while 22 patients (12 treated and 10 untreated) were persistently negative for ANA and SMA. At the time of investigation, 13 patients were ANA positive (titer range, 1/10 to 1/160; median: 1/10), 20 were SMA positive (titer range, 1/10 to 1/40; median: 1/10), and ten were ANA/SMA double positive (titer range, 1/10 to 1/40; median: 1/10) (Table I).

Two groups of HBsAg-negative pathological controls were investigated: 104 patients with other chronic liver disorders and 36 with extrahepatic autoimmune diseases (Table I).

Of the 104 patients with other chronic liver disorders, 24 had chronic HCV infection. All were HCV RNA positive (Amplipcr, Hoffmann-La Roche, Basel, Switzerland). Liver biopsy was performed in 20 patients, of whom three (15%) had histological evidence of chronic hepatitis with mild activity and 17 (85%) had chronic hepatitis with minimal activity (3). Of these, 12 were autoantibody positive at a titer ≥1/10. One was ANA positive (titer 1/10), seven were SMA positive (median titer 1/20, range 1/10 to 1/40), three were ANA (all 1/10) and SMA (1/10, 1/40, 1/40) positive, and one was SMA and LKM1 positive (titer 1/40 for both). Thirty-six patients had autoimmune liver disease: 24 autoimmune hepatitis (AIH), diagnosed according to international criteria (4) (12 ANA and/or SMA positive and 12 LKM1 positive), and 12 were ANA/SMA-positive sclerosing cholangitis (autoimmune sclerosing cholangitis, (ASC)) with characteristic cholangiographic changes (5). Autoantibody titers ≥1/10 were observed in 33 patients. Twelve patients (six AIH, six ASC) were ANA (titer range 1/40 to 1/10,240; median 1/640) and SMA double positive (titer range 1/20 to 1/2560; median 1/160), six (three AIH and three ASC) were ANA positive (titer range 1/80 to 1/5120; median 1/480), four (two AIH and two ASC) were SMA positive (1/10 in one, 1/40 in two, and 1/640 in one), and 11 were LKM1 positive (titer range 1/200 to 1/10,240; median 1/640). Ten children with Wilson’s disease, 11 with Alagille’s syndrome, 11 with α1 antitrypsin deficiency (AATD) (PIZZ phenotype), and 12 adults with primary biliary cirrhosis (PBC) (all AMA positive; titer range 1/160 to 1/5,120; median 1/800) were also tested. Six patients with Wilson’s disease were autoantibody positive, four for ANA (1/10 in one, 1/40 in two, and 1/640 in one) and two for SMA (both at 1/10).

The 36 patients with extrahepatic autoimmune diseases included 12 patients with systemic lupus erythematosus (SLE) (diagnosed according to the revised criteria of the American Rheumatologic Association (6); 11 ANA positive), 12 with AIT (all positive for Abs to anti-thyroglobulin (titer range 1/400 to 1/6,553,600; median 1/1000) and/or anti-thyroid microglobulin (titer range 1/256,600 to 1/1,638,400; median 1/25,600) by passive particle agglutination (Fujirebio, Tokyo, Japan); four ANA positive, two SMA positive) and 12 with polymyositis (7) (5 ANA positive (four nucleolar pattern), two SMA positive, and one AMA positive).

Sera from 24 healthy children (median age 9 yr (2–14); 16 boys) age-matched with the children with chronic HBV infection were tested as controls.

### Protein database search

The PIR and SWISSPROT (Genetics Computer Group, Madison, WI) protein databases were scanned using the motif search programme FINDPATTERNS to search for local sequence homology between hepatitis B virus, subtype adr, and human nuclear and smooth muscle proteins. The complete sequences of the HBV proteins, pre-S, HBsAg, HBpre-C, HBcAg, HBX, and HBV-DNA polymerase were serially divided into twelve amino acid segments, each overlapping the preceding segment by six amino acids. The resulting set of twelve amino acid sequences was used to scan the PIR and SWISSPROT protein databases for sequence homology with human nuclear and smooth muscle proteins using the FINDPATTERNS motif search algorithm. Human homologues and their corresponding HBV sequences, sharing at least 70% homology, were selected for synthesis (8, 9).

### Peptide synthesis

Twenty-mer amino acid peptides containing the relevant homologues between the HBV DNA polymerase and the antigenic targets of ANA and SMA (Fig. 1) and a 20-mer irrelevant control peptide, HEDYVNQSLRPTEPLEIVSRA, were synthesized with 9-fluorenylmethoxycarbonyl (Perkin-Elmer, Warrington, England), using an automated peptide synthesizer (431A Synergis, Perkin-Elmer). After cleavage with 5% thioanisole (Perkin-Elmer) and 2.5% ethanedithiol in trifluoroacetic acid (Perkin-Elmer) and 2.5% ethanedithiol in trifluoroacetic acid (Perkin-Elmer), peptides were precipitated in methyl-1-butyl ether (Perkin-Elmer), and the purity was checked by HPLC (Perkin-Elmer).

### ELISA

Reactivity to the control and synthetic peptides was determined by ELISA. Briefly, peptides diluted in PBS to a concentration of 50 μg/ml were coated onto 96-well ELISA microtiter plates (Flow Laboratories, Herts, U.K.) overnight at 4°C. Plates were washed three times in 1% Tween 20 (Sigma Chemical, Poole, Dorset, England), incubated with 5% BSA for 1 h, and followed by the addition of 100 μl of patient and control sera, diluted 1/100 in PBS-Tween, for 2 h at 37°C. After washing, 100 μl of horseradish peroxidase-conjugated rabbit anti-human IgG (Dako) was added, diluted 1/2000 in PBS-Tween, and incubated for 1 h at 37°C. The reaction was developed using 100 μl of substrate (0.4 mg/ml o-phenyldiamine in citrate phosphate buffer (pH 5.0) containing 4 μl of 3% hydrogen peroxide), terminated with 50 μl of 3 M H2SO4, and OD was read in a Molecular Devices (Hayward Heath, West Sussex, England) microplate reader at 490 nm. Patient and control sera were tested in triplicate. To determine a cut-off point that also takes into account non-specific binding, the highest value of the mean OD + 3SDs for the 24 normal subjects was determined as 1.5, and reaction for a given peptide

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Median Age [years [range]]</th>
<th>Sex (F/M)</th>
<th>Autoantibody Titer 1/10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chronic HBV infection</strong></td>
<td>65</td>
<td>8 (2–16)</td>
<td>26/39</td>
<td>ANA 13, SMA 20, ANA/SMA 10, LKM1 —, AMA —</td>
</tr>
<tr>
<td><strong>Chronic HCV infection</strong></td>
<td>24</td>
<td>7 (3–16)</td>
<td>12/12</td>
<td>ANA 1, SMA 8, ANA/SMA 3, LKM1 1</td>
</tr>
<tr>
<td>AIH</td>
<td>12</td>
<td>12 (4–17)</td>
<td>10/2</td>
<td>ANA 3° 2, SMA 6, ANA/SMA 1, LKM1 —, AMA —</td>
</tr>
<tr>
<td>ASC</td>
<td>12</td>
<td>5 (2–23)</td>
<td>10/2</td>
<td>ANA —, SMA —, ANA/SMA —, LKM1 11°, AMA —</td>
</tr>
<tr>
<td><strong>Wilson’s disease</strong></td>
<td>10</td>
<td>15 (8–20)</td>
<td>1/9</td>
<td>ANA 4, SMA 2, ANA/SMA —, LKM1 —, AMA —</td>
</tr>
<tr>
<td><strong>Alagille’s syndrome</strong></td>
<td>11</td>
<td>13 (2–16)</td>
<td>4/7</td>
<td>ANA 1, SMA 4, ANA/SMA —, LKM1 —, AMA —</td>
</tr>
<tr>
<td><strong>AATD</strong></td>
<td>11</td>
<td>10 (2–16)</td>
<td>7/4</td>
<td>ANA —, SMA —, ANA/SMA —, LKM1 —, AMA —</td>
</tr>
<tr>
<td><strong>PBC</strong></td>
<td>12</td>
<td>61 (47–73)</td>
<td>12/0</td>
<td>ANA 4, SMA 2, ANA/SMA —, LKM1 —, AMA —</td>
</tr>
<tr>
<td><strong>SLE</strong></td>
<td>12</td>
<td>38 (22–68)</td>
<td>11/1</td>
<td>ANA 11, SMA 11, ANA/SMA —, LKM1 —, AMA —</td>
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<tr>
<td><strong>AIT</strong></td>
<td>12</td>
<td>47 (24–64)</td>
<td>10/2</td>
<td>ANA 4, SMA 2, ANA/SMA —, LKM1 —, AMA —</td>
</tr>
<tr>
<td><strong>PM</strong></td>
<td>12</td>
<td>50 (20–67)</td>
<td>8/4</td>
<td>ANA 5°, SMA 2, ANA/SMA —, LKM1 —, AMA 1</td>
</tr>
<tr>
<td><strong>Healthy controls</strong></td>
<td>24</td>
<td>9 (2–14)</td>
<td>8/16</td>
<td>ANA —, SMA —, ANA/SMA —, LKM1 —, AMA —</td>
</tr>
</tbody>
</table>

* n, Number of patients.
* One patient was SMA- and LKM1-positive at the time of testing.
* One treated patient in each group was negative at the time of testing.
* Nucleolar pattern in four patients and homogenous pattern in one using HEp-2 cells.
was considered positive when OD$_{490}$ (test peptide)/OD$_{490}$ (control peptide) was $\approx 1.5$ (adapted from Kemeny, Ref. 10).

To ensure consistency between assays, four sera, including two negative and two positive, were used as reference controls in each assay. Mean coefficient of variation values ranged from 1.3% to 9.7% (intraassay) and 3.7% to 13.1% (interassay).

**Inhibition studies**

Competition ELISAs were performed using both peptides and native proteins as competitor in the liquid phase: 1) HBV-pol peptide was coated onto 96-well ELISA microtiter plates (Flow Laboratories) at a concentration of 50 $\mu$g/ml and incubated overnight at 4°C. Nonspecific reactive sites were blocked by incubating with 5% BSA in PBS at 37°C for 1 h. Solutions of HBV-pol peptides, the corresponding self homologue, and the control peptide were prepared at concentrations of 10, 50, 100, 250, 500, and 1000 $\mu$g/ml in PBS. Test serum was diluted in these solutions to a final dilution of 1/100, and, following incubation at 37°C for 2 h, 100-$\mu$l aliquots of the peptide/Ab mixture were transferred to the wells of the precoated 96-well ELISA microtiter plates in triplicate. This was performed for all HBV-pol/self peptide pairs (Fig. 1). In a variation to this assay, all peptides possessing the EK[K,R]RL sequence motif were used to inhibit serum reactivity to HBV-pol99–118, PM-Scl 761–780, caldesmon 600–619, and myosin 836–855 coated onto ELISA microtiter plates. Inhibition of reactivity to PM-Scl 761–780, caldesmon 600–619, and myosin 836–855 was investigated with competitor peptides prepared at concentrations of 10, 50, and 250 $\mu$g/ml, since reactivity to HBV-pol99–118 was inhibited most efficiently over this range; 2) HBV-pol99–118 peptide was coated onto microtiter plates, and reactive sites were blocked as above. Solutions of purified native caldesmon and myosin (11, 12) isolated from sheep aorta (kindly provided by Professor Marston, National Heart and Lung Institute, London, England) and a control protein, n-fructose 1, 6 diphosphatase (Sigma) were prepared at concentrations of 1, 5, 10, 25, 50, and 100 $\mu$g/ml in PBS. Test sera were diluted in these solutions to a final dilution of 1/100, and competition ELISA was performed as in part 1, above. Ab detection was conducted under identical conditions to those described under ELISA (see above).

**Statistical analysis**

Comparison between categorical values was done using the $x^2$ test with Yates’ correction, when necessary. A $p$ value less than 0.05 was considered significant.

**Results**

**Protein database search**

The protein database search revealed six human nuclear and smooth muscle proteins with high local sequence similarity to the HBV-pol (Fig. 1). Four nuclear proteins, namely MHC class II trans-activator (13) (MHCITA), nuclear pore complex protein (14) (NPCP), nuclear mitotic apparatus (15) (NuMA), and polymyositis sclerosis Ag (16) (PM-scl), were identified with sequence similarities ranging from 75–100% with their respective HBV-pol peptides spanning 6–12 amino acid regions. Two smooth muscle proteins, myosin (17) and caldesmon (18) were identified with

**Immunoblot**

Abs to caldesmon and myosin were tested by immunoblot using the sera of HBV positive patients who had double reactivity to HBV-pol99–118 and corresponding self-homologues, myosin$_{836–855}$ or caldesmon$_{600–619}$. Caldesmon and myosin (11, 12) were loaded onto 7.5% polyacrylamide gels (Bio-Rad Laboratories, Hemel Hempstead, England) (1 $\mu$g/well); following electrophoresis, proteins were blotted onto the nitrocellulose filters in a semidry electrophoretic transfer cell (Bio-Rad Laboratories) and nonspecific binding was blocked by 5% skimmed milk in TNT buffer (10 mmol/l Tris buffer pH 8.0 containing 0.15 mmol/l NaCl and 0.05% Tween 20) for one h. Filter strips were incubated with the patients’ sera for two h at 1/200 (for myosin) or 1/300 (for caldesmon) dilution and the Ags targeted by the patients’ Abs were visualized using peroxidase-conjugated rabbit anti-human IgG (Dako) at a dilution of 1/750 for one h.

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**FIGURE 1.** Amino acid sequence homology between the hepatitis B virus DNA polymerase (HBV-pol), subtype adw, and human nuclear and smooth muscle proteins. The sequences in bold represent homologous peptides constructed for testing by ELISA. Sequence alignment of the most common HBV subtypes, adw and ayw, demonstrating extensive sequence conservation in homologous regions is given. Amino acids in standard single letter code. Colon, Identical residues. Period, Conservative substitution. Dash, Identical residues to HBV-pol adr. Underlined amino acid residues represent conservative substitutions between HBV-pol adr and other HBV subtypes.
sequence similarities ranging from 90–100% with their HBV-pol peptide homologues, within 7–10 amino acid regions. The smooth muscle homologues and the PM-scl Ag contained the amino acid peptide homologues, within 7–10 amino acid regions. The smooth sequence similarities ranging from 90 –100% with their HBV-pol in all 65 chronic HBV-infected

**Reactivity to HBV-pol peptides.** Reactivity to one of the four HBV-pol peptides was observed in all 65 chronic HBV-infected patients, as compared with 24 (23%) patients with other chronic liver disorders. Among the chronic HBV-infected patients, the frequency of binding to the individual HBV-pol peptides (HBV-pol 57–76, HBV-pol 15–34, and HBV-pol 99–118) was 65%, 55%, 55%, and 52%, respectively (Table II), with 22 (34%) patients reactive to at least three of these peptides. Reactivity to the individual HBV-pol peptides was similarly present in HBV-DNA-positive and HBV-DNA-negative patients.

Of the 24 patients with other chronic liver disorders, 19 (seven with chronic HCV infection, four with PBC, four with Alagille’s syndrome, three with LKM1 AIH, and one with AATD) were reactive to one HBV-pol peptide and five (two with LKM1 AIH, one with PBC, one with AATD, and one with ANA/SMA AIH) were reactive with two HBV-pol peptides. Of the nine patients with extrahepatic autoimmune diseases, six (three with SLE and three with AIT) were reactive with two HBV-pol peptides. Of the 24 patients with other chronic liver disorders, 19 (seven with chronic HCV infection, four with PBC, four with Alagille’s syndrome, three with LKM1 AIH, and one with AATD) were reactive to one HBV-pol peptide and five (two with LKM1 AIH, one with PBC, one with AATD, and one with ANA/SMA AIH) were reactive with two HBV-pol peptides. Of the nine patients with extrahepatic autoimmune diseases, six (three with SLE and three with AIT) were reactive with two HBV-pol peptides.

**Reactivity to nuclear peptides.** Reactivity to either the MHCIITA 642–661, NPCP 827–846, NuMA 712–731, or PM-scl Ag 761–780 was significantly more common in patients with chronic HBV infection (60; 92%) than in patients with other chronic liver disorders (20; 18%), extrahepatic autoimmune diseases (6; 17%), or healthy controls (0%) (p < 0.001 for all). Of the 60 patients with chronic HBV infection who were reactive to one of the four nuclear peptides, 21 (35%) were ANA positive at a titer ≥1/10 while 29 (65%) were ANA negative. Of the 20 patients with other chronic liver disorders, five (three with ANA/SMA positive AIH and two with healthy controls.)

| Table II. Frequency, (n (%)), of reactivity to the HBV DNA polymerase and the autoantigenic targets of ANA and SMA in patients with chronic HBV infection, other chronic liver disorders, extrahepatic autoimmune disorders, and in healthy controls. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Chronic HBV | Chronic HCV | ANA/SMA | LKM1 | ANA/SMA | Wilson’s Alagille’s Syndrome | AATD | PBC | SLE | AI T | PM |
| Controls | (n = 65) | (n = 24) | (n = 12) | (n = 12) | (n = 10) | (n = 11) | (n = 12) | (n = 12) | (n = 12) | (n = 12) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 43 (66) | 36 (55) | 37 (57) | 26 (40) | 35 (54) | 37 (57) | 35 (54) | 24 (37) | 17 (26) | 35 (54) |
| 0 | 3 (12) | 1 (8) | 0 | 6 (24) | 1 (4) | 6 (24) | 2 (8) | 6 (24) | 6 (24) |
| 0 | 0 | 0 | 2 (17) | 0 | 0 | 0 | 0 | 2 (17) | 1 (8) |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* n, Number of patients.
Wilson’s disease) were ANA positive and 15 were ANA negative (four with chronic HCV, three with LKM1 AIH, two with Wilson’s disease, two with PBC, two with Alagille’s syndrome, and two with AATD). Of the six patients with extrahepatic autoimmune diseases, five (three with SLE and two with AIT) were ANA positive and one (with AIT) was ANA negative.

**Reactivity to smooth muscle peptides.** Reactivity to either caldesmon_600–619 or myosin_836–855 was also significantly more common among patients with chronic HBV infection (33/65; 51%) than in patients with other chronic liver disorders (12/104; 12%), extrahepatic autoimmune diseases (6/36; 17%), or healthy controls (0%) (p < 0.001 for all). Of the 33 chronic HBV-infected patients who were reactive either to caldesmon_600–619 or myosin_836–855, 23 (70%) were SMA positive while 10 (30%) were SMA negative. Of the 12 patients with other chronic liver disorders reactive to caldesmon_600–619 or myosin_836–855, six were SMA positive (four with ANA/SMA ASC, two with ANA/SMA AIH) and six were SMA negative (two with ANA/SMA AIH, two with chronic HCV, one with ANA/SMA ASC, and one with PBC). None of the six patients with extrahepatic autoimmune diseases (three with SLE and three with AIT) was SMA positive.

**Double reactivity to HBV-pol peptides and self homologues.** Double reactivity to the HBV-pol peptide and to the MHCIITA_642–661, NPCP_827–846, NuMA_712–731, or PM-scl Ag_761–780 was noted in 19 (29%), 20 (31%), 19 (29%), and 26 (40%) patients with chronic HBV infection, respectively (Table II), as compared with four (4%) of the patients with other chronic liver disorders (two with HCV infection, one with Alagille’s syndrome, one with

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**FIGURE 2.** A-F, Inhibition of Ab binding to the HBV-pol peptide by preincubation of serum with the HBV-pol peptide (□), corresponding self homologues (○), and control peptide (▲). Ab binding is represented as a percentage (± SEM) of binding to the HBV-pol peptide in the absence of a competitor peptide. Where error bars are not shown, errors are smaller than the data symbols. A, □, HBV-pol_633–652; ○, MHCIITA_642–661; ▲, HBV-pol_87–76; ○, NPCP_827–846; C, □, HBV-pol_15–34; ○, NuMA_712–731; D, ○, HBV-pol_10–116; ○, PM-scl Ag_761–780; E, □, HBV-pol_10–116; ○, caldesmon_600–619; F, □, HBV-pol_10–116; ○, myosin_836–855.
ANA positive showed double reactivity to the NuMA 712–731 and double reactivity to myosin 836–855 or caldesmon 600–619 and their corresponding HBV-pol peptide was similarly observed in ANA positive and ANA negative patients (Table II). Double reactivity to the HBV-pol99–118 peptide and to myosin 836–855 and caldesmon 600–619 was observed in 17 (26%) and 22 (34%) patients, respectively, with chronic HBV infection but in none of the pathologic or healthy controls (p < 0.001 for all) (Table II). Among the patients with chronic HBV infection, the double reactivity to myosin 836–855 or caldesmon 600–619 and their corresponding HBV-pol peptide was significantly more common in SMA-positive than SMA-negative patients (p = 0.019 for myosin, p = 0.011 for caldesmon) (Table III).

Inhibition studies
Ab binding to individual HBV-pol peptides was inhibited 50–90% by preincubation with the HBV-pol peptide or self homologue. No inhibition was observed with the control peptide (Fig. 2). Ab binding to the HBV-pol99–118 peptide was also inhibited by 60–70% by preincubation with the native proteins (caldesmon and myosin) but not with the control protein (Fig. 3).

Inhibition studies were also performed between HBV-pol99–118 and PM-scl Ag 761–780, caldesmon 600–619 or myosin 366–855 since these peptides contain the common amino acid motif EK[K,R]RL. The reactivity to HBV-pol99–118 was inhibited by 60 to 80% by preincubation with PM-scl Ag 761–780, caldesmon 600–619 or myosin 366–855 but not with the control peptide (Fig. 4). Inhibition of reactivity to HBV-pol99–118, PM-scl Ag 761–780, caldesmon 600–619 and myosin 366–855 by EK[K,R]RL-containing peptide homologues exhibited similar kinetic profiles (Fig. 4).

Immunoblot
Caldesmon. Using caldesmon as target Ag, two closely spaced bands of roughly equal intensity at ~120 kDa and ~130 kDa position (11, 12) were produced by seven of ten sera from HBV-positive patients double reactive to HBV-pol99–118 peptide and self homologue caldesmon 600–619 by ELISA (Fig. 5A). Sera of 30 subjects unreactive to caldesmon 600–619 peptide by ELISA (ten HBV-positive patients, ten pathological controls (two ASC, three AATD, three Wilson’s disease, two ANA/SMA AIH) and ten healthy controls) were also unreactive to native caldesmon.

Myosin. Using myosin as target Ag, a ~200-kDa band was produced by seven of ten sera from HBV-positive patients double reactive to HBV-pol99–118 peptide and self homologue myosin 366–855 by ELISA (Fig. 5B). Sera from 30 subjects (ten HBV-positive patients, ten pathological controls (four AATD, three ASC, two ANA/SMA AIH, and one Wilson’s disease) and ten healthy controls) unreactive to myosin 366–855 peptide by ELISA were also unreactive to native myosin.

Discussion
Autoantibodies to smooth muscle and nuclear components are commonly detected in patients with chronic HBV infection (1). These autoantibodies do not seem to be induced or influenced by IFN-α therapy, but appear to be a consequence of HBV infection. In an attempt to explain the occurrence of ANA and SMA in chronic HBV infection, we explored the possibility that immunological cross-reactivity, based on sequence similarity between the HBV and host proteins, may give rise to these autoantibodies. Since the putative cross-reactive epitopes targeted by ANA and SMA must lie within human smooth muscle or nuclear components, we searched the major protein databases for local sequence similarities between the HBV Ags and human smooth muscle and nuclear proteins. Using a computer-assisted scanning protocol, we identified six human proteins that have high local sequence similarity with the HBV-DNA polymerase: four nuclear proteins, namely, MHCIIITA (13), NPCP (14), NuMA (15), and PM-scl Ag (16), and two smooth muscle proteins, myosin (17) and caldesmon (18). The four nuclear proteins that we identified are key proteins involved in structural and regulatory functions, while the smooth muscle proteins are involved in muscle contraction.

To test whether these structural similarities of HBV-DNA polymerase and human nuclear and smooth muscle proteins produce cross-reactivity, we constructed the homologous peptides and used them as targets in an ELISA for the sera of our patients. Reactivity to at least one of the selected HBV-pol peptides was observed in all of the 65 patients with chronic HBV infection. This is in agreement with findings of Feitelson et al. (20), who detected HBV-pol reactivity to at least one of three carboxyl-terminal- and amino-terminal-derived HBV-pol peptides in 100% of HBV-infected renal dialysis patients. In the study by Feitelson et al. (20), one peptide, HBV-pol29–38, overlaps with HBV-pol15–34, tested in this study. However, these authors report HBV-pol29–38 reactivity in only 35% of HBV-infected patients, while HBV-pol15–34 reactivity was detected in 66% of patients in the present study. This discrepancy suggests that HBV-pol15–34 encompasses a more complete Ab epitope than does HBV-pol29–38.

Ab reactivity to all of the human nuclear and smooth muscle peptide homologues was remarkably restricted and strongly associated with chronic HBV infection, providing a clear indication that these Abs are generated specifically as a consequence of HBV infection. Double reactivity to HBV-pol peptide and self homologue was observed almost exclusively in patients with chronic HBV infection. Ab recognition of HBV-pol/self peptide pairs was cross-reactive, as preincubation with self homologue inhibited Ab binding to the corresponding HBV-pol peptide, thus providing a mechanism for the simultaneous recognition of homologous viral/
self peptide pairs. Significantly, Ab cross-reactivity between HBV-pol99–118 and the smooth muscle-derived Ags, myosin836–855 and caldesmon600–619, was associated with SMA positivity in patients with chronic HBV infection, suggesting a central role for HBV-pol99–118 in the generation of SMA. This contention is further supported by two observations: first, the ability of the native proteins, myosin and caldesmon, to inhibit Ab binding to the homologous HBV-pol99–118 peptide; and second, the ability of the sera from HBV-positive patients double reactive to HBV-pol99–118 peptide and corresponding self homologues (myosin836–855 and caldesmon600–619) to give particularly strong signals for native caldesmon and myosin on immunoblot. Similarly, double reactivity to one of the nuclear targets, NuMA, and corresponding HBV-pol peptide (HBV-pol15–34) tended to be more common in HBV-positive patients who are ANA positive. Why sera double reactivity to HBV-pol and the other nuclear proteins (NPCP827–846, MHCIIA712–731, and PM-scl Ag761–780) does not correspond to positivity for ANA by immunofluorescence remains to be determined. This may be due to intrinsic differences in the detection of nuclear epitopes by immunofluorescence and ELISA, immunofluorescence being a technique of lower analytical power. Moreover, since the protein databases screened do not contain the entire set of human nuclear proteins, the possibility that as yet uncharacterized nuclear Ags, with homology to HBV-pol, are responsible for the ANA observed by immunofluorescence cannot be discounted.

Interestingly, of the six nuclear and smooth muscle proteins identified to have homology to HBV-pol99–118, three share in common the amino acid sequence motif EK[K,R]RL with HBV-pol99–118. Since one of these motif-containing peptides is derived from the nuclear autoantigen PM-sclerosis (PM-scl Ag761–780), it is possible that ANA and SMA may arise, at least in part, as a consequence of immunological cross-reactivity with a single determinant of HBV-pol99–118. Specific inhibition of Ab reactivity to HBV-pol99–118 peptide by all three motifs containing self homologues demonstrates this cross-reactivity in vitro, providing a basis for the speculation that a similar mechanism may give rise to both ANA and SMA in vivo. Inhibition of reactivity to PM-Scl Ag761–780, caldesmon600–619, and myosin836–855 by all EK[K,R]RL-containing peptide homologues suggests that the cross-reactivity

FIGURE 4. Specific inhibition of Ab binding to (A) HBV-pol99–118, (B) PM-scl Ag761–780, (C) caldesmon600–619, and (D) myosin836–855, by preincubation of serum with HBV-pol99–118 ( ), PM-scl Ag761–780 (△), caldesmon600–619 (●), myosin836–855 (○), and control peptide (▲). Ab binding is represented as a percentage of binding to the HBV-pol peptide in the absence of a competitor peptide. SEM for all datapoints is less than ±4%.
observed operates on the EK[K,R]RL motif, since this is the only region of similarity shared among the peptides.

The epitopes identified in this study are likely to be “linear” in nature since patient sera recognizing HBV-pol99-118 cross-react with both peptide and native myosin and caldesmon in ELISA, while the same sera recognize native myosin and caldesmon by Western blot, a denaturing assay. Moreover, the relatively short peptides used in this study (20 mer) would not be expected to readily adopt stable conformations analogous to their tertiary structure in the corresponding folded proteins. However, a “conformational” component in Ab reactivity to these peptide epitopes cannot be ruled out, since the reaction conditions in ELISA do not entirely preclude the adoption, by Ag, of native tertiary structure. Hence, a “conformational” component in Ab reactivity to these peptide epitopes cannot be totally ruled out, since the reaction conditions in ELISA do not entirely preclude the adoption, by Ag, of native tertiary structure.

Our finding of cross-reactive Ab responses between HBV-pol peptides and homologous regions of human nuclear and smooth muscle proteins suggests that these autoantibodies may have arisen as an inappropriate evolution of the anti-HBV-pol immune response, to include antigenically similar nuclear and smooth muscle proteins. Thus, “mimicry” between viral and “self” Ags may lead to failure of tolerance to self components, resulting in autoimmunity (21). In a rabbit model (22), molecular mimicry between the HBV-pol66–75 and the immunodominant region of myelin basic protein (MBP), the major autoantigen in multiple sclerosis, gives rise to autoimmunity, illustrating the potential for HBV-pol to break tolerance and induce autoimmunity through a cross-reactive mechanism. In that study, rabbits immunized with HBV-pol66–75 generated cross-reactive humoral and cellular immune responses to MBP and developed central nervous system lesions histologically similar to those seen in multiple sclerosis.

It is important to note that the cross-reactive Abs detected are of the IgG isotype, implicating T cell “help” in the generation of these autoantibodies. Since T and B cell epitopes frequently overlap, it is possible that the cross-reactive humoral epitopes identified may also serve as T cell epitopes (23). Thus, the emergence of ANA and SMA may be the ultimate manifestation of molecular mimicry at the level of the T cell, giving rise to a T-dependent and class-switched cross-reactive humoral response. Indeed, strong evidence for molecular mimicry at the level of the T cell, as a mechanism for the development of autoimmunity, has recently been reported in experimental autoimmune encephalitis, the animal model for multiple sclerosis (24). T cell cross-reactivity between various viral and bacterial peptides and the immunodominant T cell epitope of MBP was demonstrated at the clonal level.

In summary, the immunological cross-reactivity between HBV-pol and homologous regions of nuclear and smooth muscle proteins demonstrated in this study introduces the intriguing possibility that the host anti-HBV-pol response may contribute to the generation of ANA and SMA in chronic HBV infection through molecular mimicry.

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References


