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A recombinant topoisomerase I ELISA: screening for IgG, IgM and IgA anti-topo I autoantibodies in human sera

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SUMMARY

An ELISA for the detection of anti-topoisomerase I autoantibodies in sera from patients with suspected or manifest rheumatic diseases is described. The antigen source used in this assay consists of a recombinant protein containing the last 695 C-terminal amino acid residues of human topoisomerase I (topo I). The sensitivity of the assay was 61%, while the specificity was more than 98%. Using this ELISA, 47 sera from scleroderma patients and immunopositive for anti-topo I antibodies, were screened for the presence of the isotypes IgG, IgA and IgM to topo I. Our finding that relatively high levels of IgA antibodies to topo I are present in most of the sera tested is consistent with the results of Hildebrandt *et al.* [1]. In addition, it is demonstrated that the IgG and IgA antibodies in a serum may recognize different epitope regions on the topo I polypeptide.

Keywords topoisomerase I systemic sclerosis epitope IgA autoantibodies ELISA

INTRODUCTION

Anti-nuclear autoantibodies (ANA) are important diagnostic and prognostic markers in patients with connective tissue diseases. Information concerning the antibody specificity may allow valuable considerations in differential diagnosis (reviewed in [2]). Autoantibodies against DNA topoisomerase I (topo I) in sera of patients with systemic sclerosis (SSc; scleroderma), usually detected by immunodiffusion or by the immunoblotting technique using thymus- or HeLa-cell extracts as antigen source, have been described by several investigators [3–10].

Recently, the expression of a cDNA clone encoding 91% of human topo I in *Escherichia coli* was reported [11]. This clone, designated as HTopoA, was brought to expression using the pET-3 expression vector system [12]. The recombinant protein (HTopoA protein) carried 695 carboxyl-terminal amino acids of topo I fused to 18 amino acids encoded by vector ànd linker sequences. The usefulness of the HTopoA protein in routine detection of anti-topo I antibodies in sera from patients with suspected or manifest rheumatic diseases was demonstrated by showing that sera from patients with a definite diagnosis SSc reacting with HeLa topo I, all reacted with the recombinant HTopoA protein as well. By expressing different fragments of HTopoA, it was possible to assign at least three different autoimmune epitope regions on the HTopoA protein.

In this study a sensitive ELISA is described for the detection of anti-topo I autoantibodies in patient sera using the recombinant HTopoA protein as antigen source. Using this ELISA, relatively high titres of IgA antibodies against topo I were detected in sera from SSc patients. In addition, it is demonstrated that the IgG and IgA anti-topo I autoantibodies may recognize different epitope regions on the topo I protein.

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PATIENTS AND METHODS

Sera

Most patient sera were obtained from the Department of Rheumatology of the University Hospital St. Radboud, Nijmegen, The Netherlands. Some additional sera were received from hospitals in Enschede, Utrecht, Deventer and Groningen, The Netherlands.

Expression of topo I fusion proteins

Expression of the cDNA's HTopoA, HTopoB and the A-sequence was performed as previously described [11]. Briefly, the HTopoA protein (*ca* 74 kD) was expressed as a fusion protein with 18 amino-terminal amino acids not related to the topo I sequence, the A-fragment (*ca* 33 kD) was expressed with four amino-terminal and 21 carboxyl-terminal amino acids not related to the topo I sequence, while the HTopoB protein (*ca* 125 kD) was expressed as a β-galactosidase fusion protein. Control experiments showed that the sera tested in this study displayed no immunoreactivity with β-galactosidase itself (data not shown).

The recovery and purification of the HTopoA protein from induced cell cultures as well as the establishing of an ELISA were performed essentially as described [13].

Gelelectrophoresis, protein blotting and detection of antigens
SDS–PAGE and transfer of proteins from 13% polyacrylamide gels onto nitrocellulose sheets (Schleicher & Schuell filters BA85, Dassel, Germany) was performed as described by Habets *et al.* [14]. The immunoblots were treated and processed as described [15]. The antibody–antigen complexes were detected with horseradish peroxidase-conjugated rabbit immunoglobulins to total human immunoglobulin (Dakopatts, Glostrup, Denmark; P-212), diluted 1:200 in radioimmunoassay (RIA) buffer.

For detection of anti-topo I antibodies of the isotypes IgG or IgA, immunoblots containing either the HTopoA protein, the HTopoB protein or the A-fragment were split up into two parts, whereafter each part was incubated with the same panel of sera. Subsequently, the antibody–antigen complexes were detected with one of the following rabbit anti-human peroxidase-conjugated antibodies: IgG (γ-chains) (Dakopatts; P-214) diluted 1:200 in RIA buffer; IgA (α-chains) (Dakopatts; P-216) diluted 1:200 in RIA buffer.

RESULTS

Expression of the cDNA HTopoA
Recently, the expression of three cDNA clones encoding fragments of the human cDNA topo I in *E. coli* was reported [11]. The longest cDNA, designated HTopoA, covered 2091 nucleotides (695 amino acids) of the coding sequence of topo I which consists of 2295 nucleotides (765 amino acids). This cDNA was cloned into the *Bam*HI site of the pET-3b expression vector to yield pEHTopoA. After induction, BL21(DE3) harbouring pEHTopoA produced a polypeptide (HTopoA protein) with a calculated mol. wt of 84·2 kD. On SDS–PAGE, however, the HTopoA protein migrated with an apparent mol. wt of approximately 74 kD (Fig. 1). Besides a large amount of this protein, lysates of bacteria expressing HTopoA contained several smaller prominent polypeptides of 56, 42 and 34 kD, respectively, that could not be detected in lysates of the induced wild type [11]. These smaller proteins could be the result of premature termination of translation or caused by translation initiation at internal AUG codons on the HTopoA mRNA. Another possibility is that these proteins are distinct proteolytic degradation fragments of the 74-kD HTopoA protein.

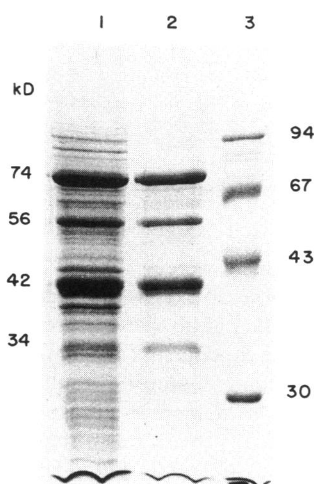

Fig. 1. The HTopoA protein produced by the pET-3 expression vector system. Lane 1: total cell lysate of induced *E. coli* BL21(DE3) containing pEHTopoA, resulting in the production of the HTopoA protein (74 kD) and three smaller polypeptides of approximately 56, 42 and 34 kD; lane 2: partially purified HTopoA protein using the Zwittergent detergent method [13] from a total cell lysate as shown in lane 2; lane 3: mol. wt markers in kD.

The HTopoA protein was partially purified using the Zwittergent detergent method (see Fig. 1, lane 2) and used as antigen source in an ELISA for detection of anti-topo I antibodies in patient sera.

Reactivity of autoimmune sera with the HTopoA protein
Serum samples from 77 patients who fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) classification criteria for definite SSc [16] with diffuse cutaneous involvement were analysed on both immunoblots and by an ELISA using the HTopoA protein as antigen source and peroxidase-conjugated rabbit immunoglobulins to total human immunoglobulin as secondary antibodies. On the immunoblot 47 sera reacted with all four proteins of 74, 56, 42 and 34 kD. In addition, with each of the 47 sera a positive reaction was obtained in the ELISA. The ELISA results are outlined in the first column of Table 1. Strong immunoreactions (optical density units (OD) at 450 nm > 2) were obtained with 29 of the 47 sera. The OD_{450} values for the other 18 sera in this group ranged from 0·7 to 2·0. A positive reaction with each of the 47 sera was also obtained when they were probed on immunoblots containing HeLa nuclear proteins (data not shown). The 30 SSc sera that gave no immunoreaction in the HTopoA ELISA or on the HTopoA immunoblot were also negative for anti-topo I antibodies when probed on a HeLa immunoblot. Considering these results the sensitivity of the HTopoA ELISA can be determined to be 61%.

Furthermore, ELISAs were performed with serum samples from patients diagnosed as having limited SSc ($n = 30$) or a non-SSc connective tissue disease ($n = 214$) and with sera from 50 healthy subjects (see Table 2). Among these control sera five were positive for anti-topo I antibodies in the ELISA. These sera were obtained from three patients with Raynaud's phenomenon (RP), from one patient with RP in combination with sclerodactyly and from one patient with Sjögren's syndrome (SS). When tested on a HeLa immunoblot the three sera from the patients with RP were clearly positive, whereas the other two sera were negative. Considering these results the specificity of the ELISA is 98·3%. However, RP has been shown to be the first symptom in 70% of the patients with scleroderma and may precede the development of this disease by many years ([18], reviewed in [19]). For this reason the specificity of the HTopoA ELISA may be even higher than the calculated 98·3%.

Detection of anti-topo I autoantibodies of different isotypes
Recently, Hildebrandt *et al.* [1] showed the existence of IgA and IgM antibodies in both the anti-topo I and anti-centromere immune response. For detection of anti-topo I antibodies of the isotypes IgG, IgA or IgM in our 47 patient sera, HTopoA ELISAs were performed using isotype-specific secondary antibodies. All 47 sera were positive for IgG anti-topo I antibodies. Positive reactions for the isotype IgA against topo I were obtained with 40 sera (85%), whereas in 21 sera (45%) IgM anti-topo I antibodies could be detected (Table 1). High titres of IgG autoantibodies were most common, but high titres of IgA anti-

Table 1. Results of anti-topoisomerase I (topo I) isotype determinations of 47 anti-HeLa topo I$^+$ sera by the HTopoA ELISA

	Total immunoglobulin	IgG	IgA	IgM
OD_{450}(NS) ± 2 s.d.	0·167 ± 0·077	0·160 ± 0·033	0·157 ± 0·036	0·182 ± 0·070
Negative	0	0	7	26
2 OD_{450}(NS) < OD_{450} < 0·5	0	1	9	8
0·5 < OD_{450} < 1·0	3	6	12	11
1·0 < OD_{450} < 1·5	4	10	7	0
1·5 < OD_{450} < 2·0	11	13	6	1
OD_{450} > 2·0	29	17	6	1

Optical density units (OD) were measured at 450 nm. Values higher than twice the mean OD value of pooled normal human serum 2 OD_{450}(NS) were considered positive.

Table 2. Specificity and sensitivity of the HTopoA ELISA

Disease*	Sera (n=371)	No. of sera positive in ELISA
RA	67	0
SLE	16	0
SS	64	1
RA/SLE overlap	50	0
SS/SLE overlap	3	0
Undifferentiated connective tissue disease	5	0
RP	8	3
RP/sclerodactyly	1	1
Limited systemic sclerosis	30	0
Diffuse systemic sclerosis	77	47
Healthy controls	50	0

* Diagnoses were reached according to published criteria [17].

The ELISA results in optical density units (OD) were read at 450 nm. Values higher than twice the mean OD value of pooled normal human serum (OD_{450}(NS) ± 2 s.d. = 0·167 ± 0·064) were considered positive.

RA, Rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; RP, Raynaud's phenomenon.

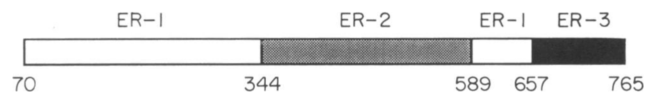

Fig. 2. Schematic representation of the different epitope regions (ER) on the HTopoA protein as recognized by sera from patients with anti-topoisomerase I (topo I) autoantibodies. The numbering of amino acids (70–765) is according to the full-length clone T1B as published by D'Arpa *et al.* [24]. ER-2 (amino acids 344–589) is represented by the A-fragment, while ER-3 (amino acids 657–765) is represented by the HTopoB protein. Amino acids 70–344 and/or 589–657 are required to form ER-1.

Table 3. Results of anti-topoisomerase I (topo I) isotype determination in 25 anti-topo I$^+$ patient sera by immunoblotting using either the HTopoA protein, the HTopoB protein or the A-fragment as antigen

	Antigen		
Isotype	HTopoA protein	HTopoB protein	A-fragment
IgG + IgA	18	7	16
IgG	6	11	7
IgA	1	1	1
Negative	0	6	1

topo I antibodies were also frequently present. In eight sera the IgA anti-topo I antibody titre even exceeded the IgG titre. In contrast, high titres of IgM anti-topo I antibodies were rare, although a considerable number of sera were found positive for this particular isotype.

By expressing different fragments of HTopoA and probing the protein products with various anti-topo I$^+$ sera on immunoblots, at least three different autoimmune epitope regions (ERs) could be allocated on the HTopoA protein ([11]; see also Fig. 2). The A-fragment (amino acids 344–589) was defined as ER-2, whereas the HTopoB protein (amino acids 657–765) was designated as ER-3. As the HTopoA protein also contains one or more epitopes which are not present on the HTopoB protein or the A-fragment, amino acids 70–344 and/or amino acids 589–657 are required to form ER-1. To obtain more information about the distribution of IgG and IgA antibodies against the three different autoimmune epitope regions on topo I, immunoblots containing either the HTopoA protein, the HTopoB protein or the A-fragment were probed with anti-topo I$^+$ sera. The antibody–antigen complexes were detected with either IgG- or IgA-specific secondary antibodies. The results of 25 sera that were screened in these experiments are summarized in Table 3. Figure 3 shows the immunoblotting results of some of these sera. When using the A-fragment as antigen source, most of the sera stain an additional protein migrating just below the A-fragment. Like the 56, 42 and 34 kD HTopoA proteins, this smaller antigen may be the result of premature termination of translation or translation initiation at internal AUG codons on the A-sequence mRNA or may be caused by proteolytic degradation of the A-fragment.

As shown in Table 3, several reaction patterns were obtained, indicating that the various sera recognized different epitopes. In four sera (16%) antibodies of both the IgG and IgA

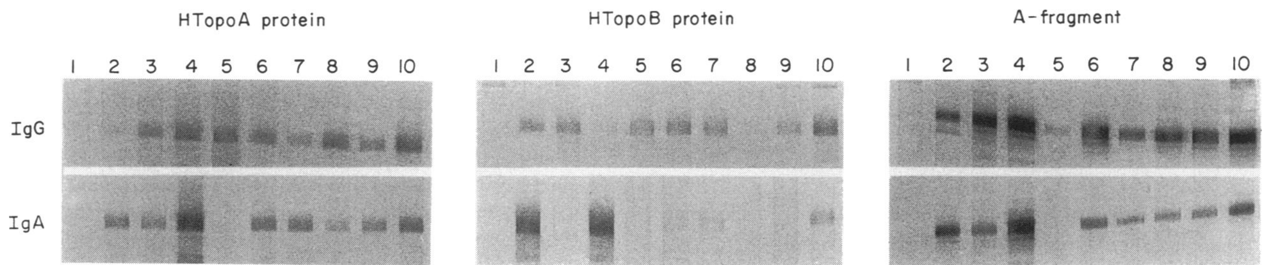

Fig. 3. Detection of IgG and IgA antibodies against the HTopoA protein (*ca* 74 kD), the HTopoB protein (*ca* 125 kD) and the A-fragment (*ca* 33 kD). Immunoblots containing these recombinant proteins were probed with pooled normal human serum (lane 1) or a systemic sclerosis (SSc) serum (lanes 2–10). These immunoblots suggest that the amounts of IgG and IgA antibodies against the three recombinant proteins differ remarkably.

isotype were detected to each of the three topo fragments, while in two sera (8%) only IgG antibodies to the three proteins were found. Five sera (20%) were positive for IgG and/or IgA antibodies to both the HTopoA protein and the A-fragment, but not the HTopoB protein. Nineteen of the 25 sera (76%) contained detectable amounts of IgA antibodies to the HTopoA protein, while in 17 of the 24 sera (71%) IgA antibodies to the A-fragment were present. In contrast, in only eight of the 19 sera (44%) could IgA antibodies to the HTopoB protein be detected.

DISCUSSION

In this study the development of a sensitive ELISA using a cloned topo I fragment (HTopoA protein) as antigen is described. Furthermore, this is the first report in which it is shown that the epitopes on the autoantigen topo I recognized by the different isotypes of autoantibodies do not necessarily have to be the same.

The HTopoA protein with an apparent mol. wt of 74 kD was recognized by 100% of the topo I$^+$ patient sera, the last selected by their reactivity with topo I on HeLa immunoblots. This suggests that although HTopoA contains only 91% of the entire topo I sequence, the HTopoA protein bears most, if not all, major autoimmune epitopes. However, as the HTopoA protein on the immunoblot and probably also in the ELISA is denatured, one cannot rule out the possibility that conformational epitopes on the HTopoA protein are missed. Nevertheless, the ELISA with recombinant topo I may be regarded as an advance in screening patient sera for the presence of anti-topo I antibodies. In addition, the presented data on the presence of anti-topo I antibodies in patient sera show that the HTopoA ELISA can be considered as specific for diffuse scleroderma.

In studying the expression of IgG, IgM and IgA autoantibodies in the anti-topo I and anti-centromere immune responses by ELISA, immunoblotting and immunofluorescence, Hildebrandt *et al.* [1] reported a high frequency of IgA anti-topo I antibodies in patient sera. These investigators used an ELISA in which the antigen source consisted of topo I purified from calf thymus tissue. Among the 17 anti-topo I$^+$ sera tested in their ELISA, 16 (94%) appeared to be positive for IgA anti-topo I antibodies. In testing Dutch anti-topo I$^+$ sera using the HTopoA ELISA, 40 of the 47 sera (85%) turned out to be positive for IgA antibodies against topo I. The frequency of IgM antibodies against the HTopoA protein in our sera was lower (45%) than the frequency of 65% reported by Hildebrandt *et al.* [1]. As the sera used in our experiments may have been thawed/frozen several times, it is difficult to evaluate the significance of both the frequency and the titres of IgM anti-topo I antibodies, since complex formation followed by precipitation and/or degradation of the IgM immunoglobulins may have reduced the titres of these antibodies.

Furthermore, our study shows that there may be considerable differences between the titres of IgG and IgA antibodies against each of the three recombinant topo fragments (compare for instance the IgG and IgA reactions of sera 2 and 3 with the HTopoB protein in Fig. 3). Although no consensus was found for the IgG and IgA immunoreaction patterns of these sera with the three proteins, IgA antibodies against the C-terminal part of topo I (i.e. the HTopoB protein) were less frequently detected than IgA antibodies against other parts of the protein.

We have still not been successful in developing ELISAs using the HTopoB protein and the A-fragment as antigens with sensitivities comparable to the HTopoA ELISA. For this reason it was not possible to quantify the immunoreactions of the IgG, IgA and IgM antibodies with the three recombinant proteins. Nevertheless, the data presented in this study allow the conclusion that the IgG and IgA anti-topo I antibodies in a serum may recognize different epitopes.

It is tempting to speculate that the autoimmune response to topo I is originated by molecular mimicry. This theory postulates that an immune response mounted by the host against a specific determinant of an infecting agent such as a virus may cross-react with the mimicked host sequence, leading to autoimmunity and, in some cases, tissue injury and disease [20]. Concrete experimental data to support this theory are few. An interesting finding in this respect has been reported by Maul *et al.* [21], who showed that antibodies from some patients with diffuse SSc cross-reacted with a synthetic peptide that had a 6 amino acid sequence match with the mammalian retroviral $p30^{gag}$ protein. One of the main features of scleroderma is endothelial abnormality in capillaries, including gaps and endothelial hyperplasia [22,23]. Consequently, the presence of IgA antibodies to topo I might be the result of an increased exposure to environmental antigens due to endothelial injury of the gastrointestinal tract. One possibility to verify such a mechanism would be to follow the level and composition of the IgA anti-topo I antibodies longitudinally during the course of the disease.

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