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Molecular Characterization of the Humoral Response to the 41-Kilodalton Flagellar Antigen of *Borrelia burgdorferi*, the Lyme Disease Agent

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The earliest humoral response in patients infected with *Borrelia burgdorferi*, the agent of Lyme disease, is directed against the spirochete's 41-kDa flagellar antigen. In order to map the epitopes recognized on this antigen, 11 overlapping fragments spanning the flagellin gene were cloned by polymerase chain reaction and inserted into an *Escherichia coli* expression vector which directed their expression as fusion proteins containing glutathione *S*-transferase at the N terminus and a flagellin fragment at the C terminus. Affinity-purified fusion proteins were assayed for reactivity on Western blots (immunoblots) with sera from patients with late-stage Lyme disease. The same immunodominant domain was bound by sera from 17 of 18 patients. This domain (comprising amino acids 197 to 241) does not share significant homology with other bacterial flagellins and therefore may be useful in serological testing for Lyme disease.

Lyme disease is an inflammatory disorder mediated by the spirochete *Borrelia burgdorferi* and transmitted by infected *Ixodes* ticks (3). The most specific manifestation of Lyme disease is erythema migrans (ECM), which is seen in most cases, followed by involvement of the joints, nervous system, or heart (19). It is now clear that the disease is widespread, being found in various areas of the United States, Europe, and Australia.

Infection with *B. burgdorferi* induces a strong humoral response. In humans, antibodies are first formed against the 41-kDa flagellar protein, and then in later stages, antibodies to the outer surface proteins OspA and OspB, among others, can be found (8). Since the flagellar antigen is detectable early in infection, interest has arisen in using this antigen as a more specific diagnostic test for infection (5, 10). It has been found, however, that conserved epitopes on the flagellar proteins of *Borrelia* spp. and other spirochetes exist (6). Indeed, sera from patients with treponemal disease yield false-positive reactions on standard Lyme disease serologic tests (14, 15). It is suspected that this cross-reactivity derives from common epitopes present on *Borrelia* spp. and other spirochetes, either *Treponema pallidum* in the case of syphilis or other commensals in the case of control human subjects. Interestingly, it has recently been shown that antiflagellar antibodies from certain Lyme disease patients are cross-reactive with human nervous system tissue (18). Though it is clear that the flagellar antigen is immunodominant early in infection, it is not fully understood which epitopes on the molecule are recognized. In this article, we describe the several epitopes present on the 41-kDa antigen recognized by sera from late-stage Lyme disease patients. To do this, we have used a new molecular genetic approach whereby fragments of the flagellar protein have been generated by the polymerase chain reaction (PCR) and expressed as fusion proteins in *Escherichia coli*.

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MATERIALS AND METHODS

Recombinant 41-kDa antigen and its fragments. The 41-kDa flagellar antigen was cloned from *B. burgdorferi* N40 (2) by PCR by using the synthetic oligonucleotide primers shown in Fig. 1, whose sequences were derived from the sequence of the 41-kDa antigen of *B. burgdorferi* B31 (9). The 5′ primer contained an *Eco*RI restriction site, and the 3′ primer contained a *Bam*HI site. Ten nanograms of N40 DNA was used as template in 50 μl of reaction mixture in a PCR reaction performed with Amplitaq polymerase (Perkin Elmer) according to the manufacturer's instructions. Thirty cycles were performed, each consisting of a 1-min 94°C denaturation step, a 1-min 40°C annealing step, and a 3-min 72°C extension step. The PCR product was isolated by agarose gel electrophoresis and purified by electroelution onto a DEAE membrane. The purified DNA was digested with *Bam*HI and *Eco*RI and cloned into *Bam*HI- and *Eco*RI-digested pMX, which is a bacterial expression vector identical to pGEX-2T (Pharmacia) except that the polycloning region of pGEX-2T is replaced with a polycloning region containing, reading 5′ to 3′, *Eco*RI, *Sma*I, *Bam*HI, *Sst*I, *Xho*I, and *Hin*dIII restriction sites. The resulting construct was designated pMX41-N40. It encodes a fusion protein containing the glutathione *S*-transferase gene at its N terminus and the complete 41-kDa antigen from N40 at its C terminus. The fusion gene is under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter and contains stop codons in all three reading frames at the 3′ end.

A series of overlapping fragments of the 41-kDa antigen gene (see Fig. 2) were generated by PCR with 10 ng of pMX41-N40 DNA as template and the synthetic primers shown in Fig. 1. Reaction mixtures (100 μl) were run as before except that 25 cycles were performed, the annealing temperature was in some cases 50°C, and the elongation step was only 2 min. PCR products were gel purified by using GeneClean (Bio 101) according to the manufacturer's instructions or low-melting-temperature agarose (FMC) in standard procedures (17). Purified fragments were digested with *Eco*RI and *Bam*HI and subcloned into similarly di-

	5'-Oligonucleotide	3'-Oligonucleotide
Flagellin	AGAGGATCC^{1}ATGATTATCAATCATAATACA^{21}	AGAGAATTC^{1008}TTATCTAAGGAATGACAAAAC^{988}
Flagellin Fragments		
A	ATAGAATTC^{3}ATTATCAATCATAATACATCA^{24}	ATAGGATCC^{111}ATTAATTCTGTACCC^{97}
B	ATAGAATTC^{3}ATTATCAATCATAATACATCA^{24}	ATAGGATCC^{198}AGTATTTCTAGAAGC^{184}
C	ATAGAATTC^{85}AAGCTTTCTAGTGGG^{99}	ATAGGATCC^{312}ATCTGAATATGTGCC^{298}
D	ATAGAATTC^{286}CAATCAGGTAACGGC^{300}	ATAGGATCC^{519}TAAAGTCCAAGACGC^{505}
E	ATAGAATTC^{493}TCAGGGTCTCAAGCG^{507}	ATAGGATCC^{723}TGTAACATTAACAGG^{709}
F	ATAGAATTC^{589}CTTTTCTCTGGTGAG^{603}	ATAGGATCC^{723}TGTAACATTAACAGG^{709}
G	ATAGAATTC^{589}CTTTTCTCTGGTGAG^{603}	ATAGGATCC^{819}TCTATTTTGGAAAGC^{805}
H	ATAGAATTC^{682}GCACCTTCTCAAGGC^{696}	ATAGGATCC^{819}TCTATTTTGGAAAGC^{805}
I	ATAGAATTC^{682}GCACCTTCTCAAGGC^{696}	ATAGGATCC^{933}ACTATTAGTTGTTGC^{919}
J	ATAGAATTC^{778}ATAAGTGATCAAAGG^{792}	ATAGGATCC^{933}ACTATTAGTTGTTGC^{919}
K	ATAGAATTC^{895}ACAATGACAGATGAG909	ATAGGATCC^{1008}TTATCTAAGCAATGACAAAAC^{988}

FIG. 1. Oligonucleotide primers used for PCR amplification of *B. burgdorferi* DNA. The primers encode a *Bam*HI or *Eco*RI restriction enzyme site followed by the *B. burgdorferi* gene sequences. Nucleotide positions are indicated by superscripts.

gested pMX. Subcloned fragments B, C, D, E, H, I, and K (which together span the entire flagellin gene) were sequenced in their entirety, through the 5′ junctions, by the dideoxy chain termination method. The deduced amino acid sequences of fragments C, H, I, and K were in accord with the published sequences of *B. burgdorferi* B31, GeHo (9) and CA12 (7). Fragment E contained an arginine at position 181 (position 1 is the first amino acid of the intact flagellin). The other strains had a glutamine at this position. This presumably reflects a strain difference, since the subcloned product of an independent PCR using N40 DNA as template also had an arginine at this position. Fragment B had isoleucine rather than methionine at position 1, and fragment D had glycines at positions 105 and 120. Since the product of the independent PCR reaction had an alanine at position 105 and a glutamate at position 120, the glycines are the result of PCR artifacts.

E. coli DH5α clones transformed with the recombinant plasmids were grown to an A_{600} of about 0.5 and induced to express high levels of fusion protein by the addition of IPTG to a final concentration of 1 mM. Cells were grown in the presence of IPTG for 5 h at room temperature. Cells were then harvested, taken up in 1/50 volume of phosphate-buffered saline (PBS) plus 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride, and sonicated, and cellular debris was pelleted by centrifugation for 10 min in a microcentrifuge. All fusion proteins were present in the supernatant except for that containing the full-length 41-kDa antigen. The pellet containing this protein was solubilized in 2% sodium dodecyl sulfate (SDS) and not purified further. The remaining, soluble proteins were affinity purified on a Sepharose 4B-glutathione column (Pharmacia) according to the manufacturer's instructions.

Sera. All patients except for patients 11, 12, and 14 were seen at the Lyme disease clinic of the Department of Rheumatology, Yale University School of Medicine. All were serologically positive on a standard enzyme-linked immunosorbent assay with whole sonicated *B. burgdorferi* as antigen. Patients 5, 8, 16, and 17 had had ECM. Patients 2, 4, 5, 6, 7, 8, 10, and 11 had had chronic arthritis for >6 months. Patient 14 had peripheral neuritis, patient 16 had Bell's palsy, patient 17 had severe headache, and patient 18 had encephalopathy. Patients 1, 3, 9, 12, 13, and 15 had arthritis.

Immunoblot analysis. Recombinant fusion proteins were boiled for 5 min in sample buffer containing 2% SDS, 100 mM dithiothreitol, and 50 mM Tris HCl (pH 7.0); run on 12.5% SDS-polyacrylamide gels; and electroblotted onto nitrocellulose by using a Hoefer Transphor apparatus. Filters were stained with Ponceau S in 10% trichloroacetic acid, destained in H_2O, blocked for 1 h with 5% nonfat dry milk in PBS–0.02% sodium azide, and allowed to react for 2 h with patient sera diluted 1:300 in blocking solution, which had been preabsorbed overnight with affinity-purified glutathione *S*-transferase linked to cyanogen bromide-activated Sepharose 4B. Unbound antibody was removed by washes with PBS, PBS–0.05% Nonidet P-40, and then PBS again. Filters were then incubated for 1 to 2 h with 0.5 μCi of ^{125}I-goat anti-human immunoglobulin (Ig) (Amersham) per ml (13 μCi/μg) in blocking solution. Unbound antibody was removed as before. Filters were air dried, and antibody binding was visualized by autoradiography with intensifying screens.

RESULTS AND DISCUSSION

Expression of the 41-kDa antigen and its fragments. In order to identify epitopes recognized by antibodies in the sera of Lyme disease patients, we expressed full-length and truncated forms of the *B. burgdorferi* flagellin as fusion proteins. Figure 2 shows the flagellin fragments that we analyzed. Figure 3 shows a Coomassie-stained SDS-polyacrylamide gel electrophoresis (PAGE) gel of the fusion proteins after affinity purification from IPTG-induced *E. coli*. It can be seen in Fig. 3 that the proteins are the expected sizes. The lower-molecular-weight fragments observed in some lanes are presumably proteolytic degradation products.

Identification of immunoreactive domains of the 41-kDa antigen. Approximately equal amounts of the purified fusion proteins were loaded on SDS-PAGE gels and blotted onto nitrocellulose membranes. The gel shown in Fig. 3 is typical of the gels used for blotting. The filters were then incubated with sera from selected Lyme disease patients. After incubation with sera, the filters were washed and residual bound antibody was detected with ^{125}I-labeled goat anti-human Ig antibody. Bound secondary antibody was detected by autoradiography.

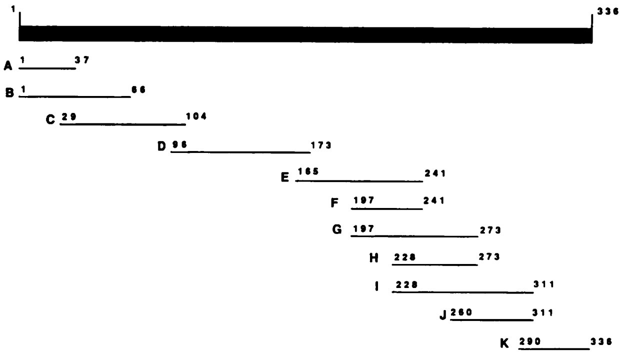

FIG. 2. Scheme of fragments used to map epitopes of the 41-kDa antigen. Fragments A through K were generated by PCR and subcloned into pMX. Numbers indicate endpoints of the fragments in amino acids.

The results are summarized in Table 1. As can be seen, a remarkably homogeneous response was observed for most patient sera. Seventeen of 18 serum samples reacted strongly to the overlapping fragments E, F, and G. Since fragments F and G are bound to approximately the same extent in all patients and since fragment H is much more weakly bound, an immunodominant domain can be mapped to amino acids 197 to 241.

In addition to this dominant domain, there are regions of generally less reactivity defined by the overlapping fragments A and B (16 of 18 patients) and by fragment D (17 of 18 patients) and encompassing amino acids 1 to 66 and 94 to 173, respectively. Some patients contain antibodies reactive with additional regions of the molecule (Table 1). Serum samples from two healthy individuals (which were also negative on the enzyme-linked immunosorbent assay) failed to react with any of the fragments (Fig. 4A and B). Moreover, we screened 10 additional control serum samples for

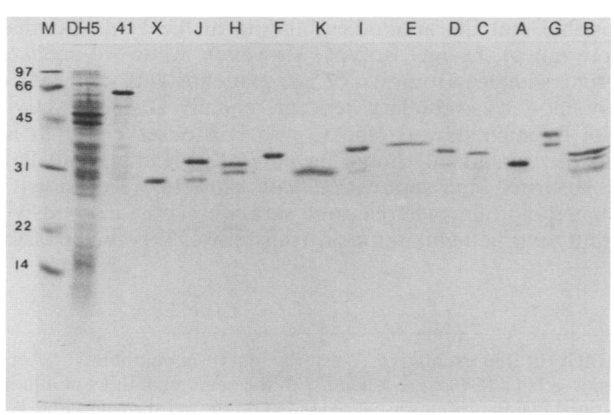

FIG. 3. Expression of fusion proteins. *E. coli* DH5α clones containing recombinant flagellin clones were induced with IPTG to express high levels of fusion proteins. The fusion proteins were affinity purified on glutathione-Sepharose 4B columns and run on an SDS-PAGE gel, and the gel was stained with Coomassie brilliant blue. Lanes: M, molecular weight markers (sizes, in kilodaltons, are indicated on the left); DH5, crude extract of wild-type DH5α; 41, partially purified fusion protein containing full-length 41-kDa antigen; X, affinity-purified glutathione *S*-transferase expressed from plasmid pMX; J through B, affinity-purified fusion proteins expressed from plasmids containing the indicated fragments of the 41-kDa antigen.

41-kDa antigen and fragment E reactivity, and all were negative (data not shown). Representative immunoblots are shown in Fig. 4. Figure 4C shows an example of the most frequent result: strong reactivity with fragments E, F, and G and weak reactivity with fragments A, B, C, and D. Panel D shows one of two patient serum samples in which binding was almost exclusively to fragments A through G. Panel E shows the results of tests with a serum sample from one of eight patients in which binding to fragments A and B was about as strong as binding to fragments E, F, and G. In no case was there significant reactivity with affinity-purified glutathione *S*-transferase. As noted in Materials and Methods, fragment D contained two amino acid substitutions. We therefore remade fragment D, sequenced it to confirm that it contained no mutations, and compared its reactivity with serum samples from five Lyme disease patients with the reactivity of the original fragment D. No difference was found (data not shown).

Patients 14, 16, 17, and 18 had neurological disease. There is evidence for cross-reactivity of antiflagellar antibodies with human neural tissue (1, 18), which suggests that these antibodies may play a role in neurological pathology. We detected no regions recognized exclusively by sera from patients with neurological symptoms. It is possible, however, that these sera recognize distinct, cross-reactive epitopes within the immunodominant flagellin domains mapped here. Alternatively, they may recognize additional epitopes not detected in this study because of, for example, a requirement for native conformation. This study would detect only antibody reactivities not lost upon denaturation and blotting.

It has been suggested that the use of purified 41-kDa antigen in immunological tests for Lyme disease may increase the specificity of such tests (10). However, the *B. burgdorferi* flagellin is known to cross-react with antibodies against other bacterial flagellins (15). It would therefore be desirable to identify non-cross-reactive immunoreactive domains of the 41-kDa antigen. A comparison of the derived amino acid sequence of the *B. burgdorferi* flagellin with those of other bacterial flagellins listed in the GenBank data base indicates regions of substantial homology to the sequences of flagellins of *E. coli* (13), *Salmonella typhimurium* (12), *Salmonella rubislaw* (20), *Serratia marcescens* (11), and *Roseburia cecicola* (16). However, these homologies are restricted to the N- and C-terminal regions of the flagellins, and, except for a short sequence in *S. marcescens* homologous to the sequence from amino acids 209 to 215 (four of seven identical residues), there are no homologies to the immunodominant domain mapped here (amino acids 197 to 241). Similarly, a comparison of the deduced *B. burgdorferi* amino acid sequence with that of the spirochete *T. pallidum* (4) reveals only a short segment of homology, from amino acids 211 to 216 (four of six identical) within the immunodominant domain, despite considerable regions of homology at the N and C termini. Thus, the predominant immunoreactivity seen in this study may be specific to *B. burgdorferi*, and peptides from this immunodominant domain may be good candidates for use in serological tests for Lyme disease. Studies to test this are currently under way. The immunoreactive fragments A and B mapped in this study contain many regions of homology to the other bacterial flagellins, and the humoral response to them may thus be relatively non-*B. burgdorferi* specific.

Recently, a study by Collins and Peltz (7) demonstrated immunoreactivity of only the amino-terminal 90 amino acids of flagellin with sera from Lyme disease patients. The strong

TABLE 1. Reactivity of Lyme disease sera with different flagellin fragments

Patient no.	Reactivity with fragment[a]:											
	A (1–37)	B (1–66)	C (29–104)	D 96–173)	E (165–241)	F (197–241)	G (197–273)	H (228–273)	I (228–311)	J (260–311)	K (299–336)	41GST (1–336)
1	−	−	−	+/−	+++	+++	+++	−	−	−	−	+++
2	+	+	+/−	+/−	+	+	+	−	−	−	−	+++
3	+/−	+/−	+/−	+/−	+++	+++	+++	−	−	−	−	++++
4	+	+	−	+	+++	+++	+++	−	−	−	−	++++
5	−	−	−	+/−	+++	+++	+++	−	−	−	−	++++
6	+	+	−	+/−	+	+	+	−	−	−	−	++++
7	+	+	−	+	−	−	++	−	++	−	−	++++
8	+	+	+/−	+	+++	+++	+++	−	−	−	−	++++
9	+	+	−	−	+	+	+	−	−	−	−	++
10	+	+	+/−	+	+++	+++	+++	+	+	−	+/−	++++
11	+	+	+/−	+	+	+	+	−	+/−	−	+	++++
12	−	+	−	+/−	+	+	+	−	−	−	−	+++
13	+	+	+/−	+	++	++	++	−	++	++	−	++++
14	+	++	+/−	+/−	+	+	+	−	+/−	+/−	+/−	+++
15	+	+	+/−	+	+	+	+	−	−	−	−	+++
16	+	+	+	+	+	++	++	−	+/−	+/−	+	+++
17	+++	+++	+	+	+	+++	+++	+/−	+/−	+/−	+/−	+++
18	+	+	+	+	+++	+++	+++	−	−	−	+	++++

[a] Fragments A through K of the 41-kDa flagellin were expressed as fusion proteins with glutathione *S*-transferase, affinity purified by binding to a glutathione-Sepharose 4B column, and analyzed by immunoblotting after SDS-PAGE. Intensity of binding to the different fragments is indicated by pluses, with ++++ being the most intense binding. Numbers under each fragment designation indicate endpoints of the fragments in amino acids. 41GST is the full-length (336-amino-acid) flagellin fused to the glutathione *S*-transferase.

reactivity to the central portion of the flagellin that we saw was not detected. Both studies used essentially the same methods, and there is no obvious difference between the patients chosen in the two studies. Collins and Peltz did use substantially larger fragments than we did (their smallest fragment was 177 amino acids), and it is thus possible that the immunodominant domain that we detected was hidden by conformational features that were absent in our fragments and survived denaturation. However, we have determined that a fragment extending from amino acids 96 to 336 is bound strongly by serum samples from two patients and by two independent pools of serum samples from about five patients each (data not shown). This is in contrast to the fragment from amino acids 90 to 336 that Collins and Peltz found not to be immunoreactive. Another possible explanation for the discrepancy between the two studies is that we used anti-human Ig as secondary reagent in immunoblots, while Collins and Peltz used anti-human IgG. It was thus possible that the antibodies binding to the central domain were not of the IgG isotype. However, when we analyzed serum samples from six of our patients with anti-IgG or protein A as secondary reagent, equally strong reactivity with fragments E, F, and G was still observed (data not shown). Finally, it should be noted that Collins and Peltz preabsorbed their sera with *E. coli* extract before using it in immunoblotting, whereas our sera were preabsorbed with affinity-purified glutathione *S*-transferase. It is thus possible

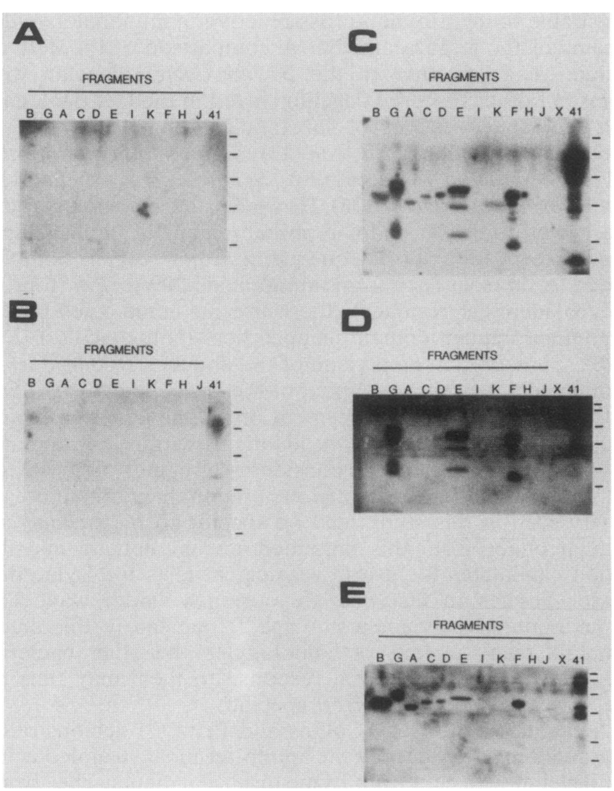

FIG. 4. Representative Western blots (immunoblots). Acrylamide gels (12.5%) were loaded with the same quantities of affinity-purified fusion proteins as were loaded on the gel shown in Fig. 3. B, G, A, C, D, E, I, K, F, H, and J indicate the fragment loaded (see Fig. 2). Lanes: X, affinity-purified glutathione *S*-transferase; 41, partially purified full-length 41-kDa antigen expressed as a fusion protein. Gels were blotted onto nitrocellulose and probed with sera. (A and B) Non-Lyme disease patients. The band in panel B, lane 41, is of higher molecular weight than the glutathione *S*-transferase–41-kDa fusion protein and represents reactivity with an *E. coli* protein(s). (C) Patient 18 of Table 1; (D) patient 5 of Table 1; (E) patient 17 of Table 1. Bars to the right of autoradiograms indicate the positions of molecular size markers as follows: (A and B) from top to bottom, 97, 66, 45, 31, and 22 kDa; (C, D, and E) sizes as for panels A and B, with the addition of a 14-kDa marker.

that antibodies recognizing the central flagellin domain were removed by the preabsorption in the Collins and Peltz study. However, in our study, sera from healthy controls, which presumably should contain anti-*E. coli* antibodies, failed to react with any flagellin fragments, suggesting that the reactivity we saw with Lyme disease sera is not due to cross-reactive anti-*E. coli* antibodies. Further study will be required to resolve the disparity between our results and those of Collins and Peltz.

In conclusion, we have mapped the human humoral response to the 41-kDa antigen of *B. burgdorferi* and found it to be remarkably homogeneous among different patients exhibiting both neurological and other manifestations of Lyme disease. This region is not homologous to any region of other bacterial flagellins and may therefore be useful for specific Lyme disease testing. We have not, however, mapped the epitopes used in the serological response to the flagellar antigen in early Lyme disease. The reactivity of sera from patients with early Lyme disease to the immunodominant regions identified here will be the subject of a future study.

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