Improved Affinity of a Human Anti-Entamoeba histolytica Gal/GalNAc Lectin Fab Fragment by a Single Amino Acid Modification of the Light Chain

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We previously produced, in Escherichia coli, a human monoclonal antibody Fab fragment, CP33, specific for the galactose- and N-acetyl-D-galactosamine-inhibitable lectin of Entamoeba histolytica. To prepare antibodies with a higher affinity to the lectin, recombination PCR was used to exchange Ser91 and Arg96 in the third complementarity-determining region of the light chain with other amino acids. The screening of 200 clones of each exchange by an indirect fluorescent antibody test showed that 14 clones for Ser91 and nine clones for Arg96 reacted strongly with E. histolytica trophozoites. Sequence analyses revealed that the substituted amino acids at Ser91 were Ala in five clones, Gly in three clones, Pro in two clones, and Val in two clones, while the amino acid at position 96 was substituted with Leu in three clones. The remaining eight clones exhibited no amino acid change at position 91 or 96. These mutant Fab fragments were purified and subjected to a surface plasmon resonance assay to measure the affinity of these proteins to the cysteine-rich domain of lectin. Pro or Gly substitution for Ser91 caused an increased affinity of the Fab, but substitution with Ala or Val did not. The replacement of Arg96 with Leu did not affect affinity. These results demonstrate that modification of antibody genes by recombination PCR is a useful method for affinity maturation and that amino acid substitution at position 91 yields Fabs with increased affinity for the lectin.

Amebiasis caused by infection with the intestinal protozoan parasite Entamoeba histolytica is a notable parasitic disease in both developing and developed countries. It has been estimated that 50 million people develop amebic colitis and extraintestinal abscesses, resulting in up to 110,000 deaths annually (18). The development of immunoprophylaxis and accurate diagnostic tools is important for the control of amebiasis. The application of monoclonal antibodies is a promising avenue of research for improvement in diagnosis.

We recently produced several human monoclonal antibody Fab fragments specific for E. histolytica in Escherichia coli by use of combinatorial immunoglobulin gene libraries constructed from the peripheral lymphocytes of a patient with an amebic liver abscess and from an asymptomatic cyst passer (1, 14, 17). One of the Fab clones, CP33, derived from the asymptomatic cyst passer, recognized the cysteine-rich domain of the heavy subunit of the galactose- and N-acetyl-D-galactosamine-inhibitable (Gal/GalNAc) lectin (12) of E. histolytica (17). This clone exhibited neutralizing activities to amebic adherence and to erythrophagocytosis. Furthermore, we produced the Fab fragment fused with alkaline phosphatase for diagnostic purposes (16).

Recombinant antibody technology makes it possible to introduce site-directed or random mutations in the original antibody gene (3–5, 13, 19). Residues in the complementarity-determining region (CDR), especially in CDR3 of both the heavy and light chains of antibody, are considered responsible for high-energy interactions with antigen. Therefore, mutations at these residues will likely abolish antigen binding. However, an increased affinity may also occur by mutation if the native residue exhibits a negative effect on the interaction. In the Kabat numbering system, CDR3 of the light chain is the amino acid segment from position 89 to 97 (6, 20). The corresponding amino acid residues in CP33 were GinGinSerTyrSer ThrProArgThr (17). When an additional 13 light chains which constitute antibectin Fabs with the heavy chain of CP33 were analyzed, high variability was observed at positions 91, 92, 94, and 96 (17). As a first step in the affinity maturation of human antibodies to E. histolytica, we attempted to modify Fab clone CP33 by single-amino-acid substitutions of Ser91 and Arg96 in the light chain.

MATERIALS AND METHODS

Site-directed mutagenesis. Site-directed mutagenesis in the light chain gene of CP33 (17) was performed by recombination PCR (7). The plasmid vector pFab1-His2, containing the light and the Fd region of the heavy chain genes, was amplified by using two sets of primers, CP33L-S91X-For (5′-CAACCTAAGCTTACGTCAACAGNNTACAGTAC-3′, where N is any nucleotide) and CP33L-S91-Rev (5′-CTGGTGGACAGTGAAGTTGCAAAATCTTC-3′), and CP33L-R96X-For (5′-AACAGAGTTACAGTACCCCTNNNACCTTCGG-3′) and CP33L-R96-Rev (5′-AGGGGTACTGTAACTCTGTTGACAGTAGTAAG-3′). The positions of these primers in the light-chain gene of CP33 are shown in Fig. 1.

To obtain high fidelity amplification, PhoyoBest DNA polymerase (Takara, Otsu, Japan) was used. Twenty-five cycles of PCR were performed as follows: denaturation at 94°C for 15 s (135 s in cycle 1), annealing at 60°C for 30 s, and polymerization at 72°C for 360 s. The PCR products were purified by agarose gel electrophoresis and by use of a Qiaex II gel extraction kit (Qiagen GmbH, Hilden, Germany). The DNA fragments were introduced into E. coli JM109 cells.

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Expression of Fabs and screening. Bacterial expression of Fabs was performed essentially as previously described (14). Each clone was cultured in 2 ml of super broth (30 g of tryptone, 20 g of yeast extract, 10 g of morpholinopropanesulfonic acid per liter [pH 7]) containing ampicillin until an optical density at 600 nm of 0.4 to 0.6 was achieved. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the bacterial cultures to a final concentration of 0.1 mM, and the cells were then cultured for a further 12 h at 30°C. The cells were harvested by centrifugation, suspended in 150 μl of phosphate-buffered saline (PBS, pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride, and then ruptured by sonication. After centrifugation of the lysates at 18,000 × g for 10 min, the supernatant was screened by an indirect fluorescent antibody test.

Indirect fluorescent antibody test. Trophozoites of *E. histolytica* HM-1:IMSS were cultured axenically in BHI-S-33 medium (2) supplemented with 10% adult bovine serum at 37°C. Trophozoites of *Entamoeba dispar* SAW1734RclAR were cultured monoxenically with *Pseudomonas aeruginosa* in BCSI-S medium at 37°C (9). These trophozoites at the logarithmic phase of growth were used in the following experiments. The indirect fluorescent antibody test was performed with formalin-fixed *E. histolytica* trophozoites, 37 positive samples were obtained. In the second screening, 10-fold-diluted samples of the *E. coli* extracts were also examined by indirect fluorescent antibody test. Fourteen samples were found to be strongly reactive with the *E. histolytica* trophozoites, which was comparable to the reactivity of the original clone CP33. Sequencing of the light-chain genes revealed that Ser91 of the light chain had been replaced by Ala in five clones, Gly in three clones, Pro in two clones, and Val in two clones. The remaining two clones showed no substitution at this amino acid, although the nucleotide sequences were changed. Interestingly, these four residues are grouped into the amino acids with nonpolar side chains. On the other hand, when the mutations were introduced at Arg96, nine clones were shown to be reactive with the *E. histolytica* trophozoites as strongly as the original clone CP33. Among these nine clones, three exhibited a replacement of Arg96 with Leu, while the other six showed no replacement.

Reactivity of the modified Fabs. We selected one clone from each group with the same mutation at Fab. These clones were cultured on a large scale to obtain Fab fragments to be purified by affinity chromatography for the His tag. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified proteins demonstrated two bands with apparent molecular masses of 24 and 25 kDa under reduced conditions (data not shown). These purified Fabs were confirmed to be reactive with the *E. histolytica* trophozoites by confocal laser scanning microscopy. Localization of bright fluorescence on the surface of the trophozoites was demonstrated by immunostaining with the modified Fabs.

RESULTS

Amino acid modifications at positions 91 and 96. Transformation of *E. coli* with two kinds of PCR products yielded more than 10⁶ colonies. Of these, 200 clones of each exchange were randomly selected and then cultured for the expression of Fabs. When *E. coli* extracts from those clones with a randomly mutated amino acid at position 91 were screened to detect Fab fragments reactive with formalin-fixed *E. histolytica* trophozoites, 37 positive samples were obtained. In the second screening, 10-fold-diluted samples of the *E. coli* extracts were also examined by indirect fluorescent antibody test. Fourteen samples were found to be strongly reactive with the *E. histolytica* trophozoites, which was comparable to the reactivity of the original clone CP33. Sequencing of the light-chain genes revealed that Ser⁹¹ of the light chain had been replaced by Ala in five clones, Gly in three clones, Pro in two clones, and Val in two clones. The remaining two clones showed no substitution at this amino acid, although the nucleotide sequences were changed. Interestingly, these four residues are grouped into the amino acids with nonpolar side chains. On the other hand, when the mutations were introduced at Arg⁹⁶, nine clones were shown to be reactive with the *E. histolytica* trophozoites as strongly as the original clone CP33. Among these nine clones, three exhibited a replacement of Arg⁹⁶ with Leu, while the other six showed no replacement.
Table 1. Association and dissociation constants of the binding of modified human Fab fragments to the cysteine-rich domain of the Gal/GalNAc lectin heavy subunit of *E. histolytica*, measured by surface plasmon resonance

| Fab | Amino acid change in light chain | $K_a$ (1/M) | Increase compared to CP33 (fold) | $K_d$ (M) | Decrease compared to CP33 (fold) |
|-----|---------------------------------|-------------|---------------------------------|-----------|---------------------------------|
| Clone 1 | Ser (AGC) | $5.53 \times 10^7$ | 0.8 | $1.81 \times 10^{-8}$ | 0.8 |
| Clone 2 | Pro (CCA) | $3.49 \times 10^8$ | 4.9 | $2.87 \times 10^{-9}$ | 4.8 |
| Clone 3 | Ala (GCC) | $6.38 \times 10^7$ | 0.9 | $1.57 \times 10^{-8}$ | 0.9 |
| Clone 4 | Gly (GCC) | $1.21 \times 10^8$ | 1.7 | $8.24 \times 10^{-9}$ | 1.7 |
| Clone 5 | Val (GTC) | $7.64 \times 10^7$ | 1.1 | $1.88 \times 10^{-8}$ | 0.7 |
| Clone 6 | Leu (CTG) | $4.95 \times 10^7$ | 0.7 | $2.02 \times 10^{-8}$ | 0.7 |
| CP33 | | $7.19 \times 10^7$ | 1.0 | $1.39 \times 10^{-8}$ | 1.0 |

*These values are from a previous study (17).*

The previous paper (17). The affinity of the Fabs with Ser$^{91}$Pro (clone 2) and Ser$^{91}$Gly (clone 4) was found to be approximately 4.8- and 1.7-fold higher, respectively, than that of the original Fab. However, the mutants with Ser$^{91}$Ala (clone 3) and Ser$^{91}$Val (clone 5) exhibited dissociation constants comparable to those for clone 1 or CP33. On the other hand, the affinity of the Fab with Arg$^{90}$Leu (clone 6) was comparable to that of CP33.

**Discussion**

The present study demonstrated that a single-amino-acid replacement of Ser$^{91}$ in CDR3 of the light chain could improve the affinity of CP33. A number of possible explanations for this observation occurred to us. First, the contribution of the Ser$^{91}$ residue to the interaction between antigen and antibody was considered. Ser is a polar amino acid and therefore may contribute to binding affinity by forming a hydrogen bond to the amino acid of the lectin. However, this possibility is unlikely because hydrophobic amino acids such as Ala and Val are not able to form a hydrogen bond, but the substitution of Ser with Ala or Val appeared to have a lesser effect on the binding affinity of the antibody.

Second, it was considered that Ser$^{91}$ might inhibit affinity through steric hindrance. The substitution of Ser with Pro leads to residue bending, which results in the conformational change which allowed the redistribution of the neighboring amino acids favoring the antigen-antibody interaction. The increased affinity of the antibody by the replacement of Ser with Gly supports the second consideration because Gly is the smallest amino acid and therefore is capable of reducing the steric hindrance caused by the Ser residue. Furthermore, this notion is consistent with the finding that the Ala and Val substitutions exhibited no effect on binding affinity because the sizes of Ala and Val residues are comparable to that of Ser. It is known that the effect of a mutation is not restricted to contact residues (19). Although the residue at position 91 may not react directly with antigenic molecules, it can affect the binding of residue 93 (5). Therefore, the second possibility seems more likely to be the explanation, although we cannot exclude other possibilities.

In contrast, improvement of affinity was not achieved by the single-amino-acid modification at Arg$^{90}$. Since the amino acid change from Arg to Leu is thought to be drastic, it is reasonable to expect a distinct change in binding affinity. However, this was not the case. At present, the reason is not clear. As the nucleotide sequence has not been analyzed in all clones, there is the possibility that substitutions to amino acids translated from only one genetic code were not included in the mutagenesis of this study. However, since 200 clones were examined, the probability that the Met and Trp substitutions were not included is theoretically less than 0.2%. Therefore, it appears that Arg may be the best residue in this position on the light chain.

To our knowledge, this is the first report demonstrating the modification of antibody genes by recombination PCR. Single-amino-acid substitution by this method demonstrated the feasibility of improving the affinity of the original human Fab. Further studies on modification of other residues in CDR3, including residues that contact the antigen, will contribute to improve the affinity of the human antibody and thereby improve its utility for diagnosis and immunophrophylaxis.

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