Chapter 3. RESULTS

Purification of each Hp phenotype was achieved to at least 95% homogeneity by an antibody affinity column using the procedures described previously (59). Electrophoretic pattern of each purified type on a 7% native-PAGE is shown in Fig. 1 (insert panel) consistent with the proposed structure of Hp 2-1 and 2-2 as multiple polymers. Notably, the purified Hp 2-1 also contained a basic structure of Hp 1-1.

3.1 MAb prepared against Hp 2-1 or 2-2

Due to the distinct structural orientation among the Hp phenotypes, we tested the hypothesis that each specific type mAb might be produced by immunizing Hp 2-1 (also containing 1-1 molecule to some extent) or 2-2. Table 1 summarizes the efficiency of hybridomas that possessed the anti-Hp activity. The efficiency of positive clones produced from the hybridomas for immunogen Hp 2-1 and 2-2 was 9.1 and 11.9%, respectively. All the positive monoclonals (n = 64) were determined for their chain specificity (α or β) using a SDS-Western blot analysis in the presence of reducing reagent (Fig. 2). Those mAb not reactive to α or β chain were defined as

conformation-dependent antibodies (n = 49), since they only recognized native Hp in an ELISA (Table 1). It is of interest to note that mAb prepared against Hp 2-1 produced mostly sequence-dependent antibodies (73%). On the contrary when immunizing Hp 2-2, only few mAb (10%) were directed against the Hp subunits. The results suggest that the surface of cyclic 2-2 polymers is distinct from that of linear 2-1 polymers (Table 1). As such, the unique surface structure of Hp 2-2 might create some epitopes that are not expressed on Hp 1-1 or 2-1. To address this possibility, we further tested all the positive monoclonals against each Hp type. There were two mAb (3B7 and 4H11) identified to be specific to Hp 2-1 and 2-2, but not 1-1. Both of them reacted with polymers of Hp in solution and in plasma. A typical example revealing such specificity for mAb 3B7 is shown in Fig. 3. However, in the presence of reducing reagent (β-mercaptoethanol), none of the Hp types could be recognized by 3B7 and 4H11, when compared with polyclonal antibodies used as control (data not shown). These two mAb are also defined as conformationally dependent (Table 2). It suggests that disulfide linkages play a role in maintaining the overall antigenic structure of Hp and that the specificity lies on the formation of polymers in Hp 2-1 and 2-2.

We further confirmed the specificity of mAb 3B7 and 4H11 using an ELISA,

the immunoreactivity of Hp 2-1 and 2-2 were almost indistinguishable and significantly greater than that of 1-1 by 15-20 times as judged by 50% of maximal binding (Fig. 4). Slight immunoreactivity in Hp 1-1 was seen, which may be rationalized as some of the epitope being exposed during the immobilization or partially expressed on the surface of Hp 1-1 regardless of the immobilization. Moreover, other conformation-dependent mAb were also characterized and exhibited differentially higher binding to Hp 2-1 and 2-2 than to 1-1. Typical examples for other conformation-dependent mAb (4E11 and 4G1) are depicted in Fig. 4 (Table 2).

3.2 Monoclonal antibody specific to Hp α chain of Hp reacted equally among Hp phenotypes

In light of the diverse immunoreactivity in Hp types, the production of mAb 3B7 and 4H11 is of significant importance. It is an example of how some of the antibody populations could underestimate the Hp levels in 1-1 subjects (Figs. 3 and 4), if used in an immunoassay. Following study of a panel of β-chain specific mAb (sequence-dependent), we found that all of them possessed slightly higher binding to Hp 1-1 than 2-1 or 2-2 (data not shown). It is therefore tempting to speculate that the surface accessibility of the antigenic

domain on the β chains of Hp 1-1 is greater than that of 2-1 and 2-2, due to the steric hindrance or reorganized antigenic structure in the formation of polymers of the latter types (Fig. 1). Therefore, one additional purpose of the present study was to identify a mAb that could react equally with Hp three types. To test this possibility, a panel of seven α-chain specific mAb was characterized. Two of them (3H8 and 2A3) identically recognized Hp 1-1, 2-1, and 2-2 when each specific Hp type was immobilized on an ELISA (Table 2). A typical dose-dependent binding curve and its specificity using the 3H8 mAb are depicted in Fig. 5.

3.3 Immunoreactivity of mAb 3H8 against Hp phenotypes in solution

To further confirm the immunoreactivity of each Hp type in solution, a competitive ELISA was conducted using Hp 1-1, 2-1, or 2-2 to simultaneously compete with the binding of each specific Hp immobilized. Again, we show that the immunoreactivity of each Hp type essentially gave indistinguishable competition curves (Fig. 6).

3.4 Determination of plasma levels of Hp in subject with Hp 1-1, 2-1, or 2-2 using α-chain specific mAb 3H8

The above results revealed a great advantage in using this unique α-chain mAb for the determination of plasma Hp levels in subjects with various Hp phenotypes. In established methods of determination, one potential problem is that hemoglobin possesses very high affinity binding to Hp with a K_a of about 10^{15} M^{-1} (2). As such, trace amount of hemoglobin in the hemolytic plasma during blood collection could drastically affect the immunoassay, if hemoglobin masked the antigenic epitope. We then investigated whether hemoglobin might block the binding of mAb 3H8, 2A3, and the other antibodies to Hp (Table 2). Typical examples are given in Fig. 7, hemoglobin inhibited the binding for some conformation-dependent mAb and also for 2A3 to some extent, but not for 3H8. We therefore used 3H8 to determine the plasma Hp levels. By a competitive ELISA, Fig. 8 shows that the Hp concentrations (mean ± SEM) were differentially higher in Hp 1-1 subjects (236 ± 27.6 mg/dl; n = 11) than in 2-1 (191 ± 20.5 mg/dl; n = 13) and 2-2 (155 ± 20.7 mg/dl; n = 13). The levels of Hp 1-1 were significantly higher than that of 2-2 ($p < 0.05$). The higher level in hp 1-1 was further confirmed by a Western blot analysis using an Hp polyclonal antibody against the pooled plasma from Hp 1-1 and 2-2 subjects (Fig. 8, insert panel).

3.5 Antigenic mapping for mAb 3H8

To give further detailed reference with respect to the uniqueness of mAb 3H8, we attempted to map out the antigenic epitope. The availability of the amino-acid sequence for this epitope may eventually offer the opportunity to generate a regional-specific polyclonal antibody. The strategy for the antigenic mapping is shown in Fig. 9. First, we checked the sequence homology by aligning mouse and human Hp α-chain sequence. MAb raised from the mice should not recognize mouse Hp α-chain, so MAb 3H8 was predicted to bind with one of the variable regions in human α-chain. Accordingly, a solid-phase peptide array containing the above variable regions and other overlapped synthetic peptides (each with 15 residues) was prepared. These peptides were directly synthesized on a nitrocellulose membrane (Fig. 9). After binding of mAb and HRP-conjugated secondary antibody, the array was developed by a chemiluminescent agent. We show that peptide 3 corresponding to residues 31-45 (or RYQCKNYYKLRTEGD) was predominantly immunoreactive; while an overlapped peptide 7 (residues 22-36) gave a partial activity. Most interestingly, the overlapped region (residues 31-39 or RYQCKNYYK) in that of human was within a variable region containing Gln-33, Lys-35, Asn-36, Tyr-37, and Lys-39 substitutes. The identified putative epitope is located

within the duplicated region of α chain. Since α2 (consisting of 142 amino-acid residues) in Hp 2-2 contains one completely identical copy of α1 (83 residues) plus one insertion of 3/4 of α1 (Fig. 9). We expected the immunoreactivity of the α2 chain to be twice of that α1. The identical immunoreactivity found among the Hp 1-1, 2-1, and 2-2 molecules was beyond our expectation.

3.6 Immunoreactivity of mAb 3H8 for recombinant α1 and 2 chains

Since the epitope is repeated twice in α2, we addressed whether the immunoreactivity was also equal between the α1 and α2 subunits by using recombinant proteins. Cloned human cDNA corresponding to each specific chain was constructed into a plasmid with expression vector pQE30 and overproduced in *E. coli* strain M15 [pREP4]. The expressed and the subsequently purified α1 and 2 proteins are shown in Fig. 10. By Western blot analysis, however, α2 gave almost twice as much immunoreactivity as compared to α1. It indicates the α2 epitopes can be bound "2x" by the 3H8 mAb. In accordance with the 3H8 binding to all three of the Hp types (Fig. 5), this result suggests that the epitope region of α2 chains is being reorganized in Hp 2-1 and 2-2 molecules which results in a decreased immunoreactivity.

Furthermore, to test the role that Tyr-32 plays in the putative antigenic

determinant (residues 31-39) for the 3H8 mAb, a recombinant Hp α1-chain with a Phe-32 substitution was prepared using a site-directed mutagenesis. The substitution completely diminished its immunoreactivity, indicating the involvement of Tyr-32 (Fig. 10).

3.7 Conformation of recombinant α1 and 2 and Hp 2-1

We analyzed the secondary structure of recombinant α1 and 2 using a circular dichroic spectrum. In Fig. 11, both recombinant α1 and 2 chains were typically random and similar in structure. The random structure of each subunit may; therefore, render a full recognition by the sequence-dependent mAb 3H8 (Figs. 9 and 10). However, the structure of whole Hp 2-1 molecule was observed to be more ordered (containing about 25% α-helical content). Thus, the 3H8 mAb may only partially bind the epitope in Hp 2-1 and 2-2 even with repeated epitopes (Figs. 4 and 5).