inhibited 61.1 and 57.7% of the binding, respectively, and the inhibition rate gradually decreased with higher dilutions. The inhibition ability was specific for KF94. The mAb2s did not affect anti-HAV human mAb KF6 binding to HAV.

Inactivated HAV inhibited KF94 binding to mAb2s (Fig. 1b). The inhibition rates were proportional to the concentration of inactivated HAV. KF94 binding to the mAb2s 94-2 and 94-7 was inhibited by 69.1 and 78.5%, respectively, at the maximal concentration of inactivated HAV. Binding of mAb2s or HAV to KF94 reduced the affinity of KF94.

Binding of mAb2 to HAV cellular receptor

Binding of mAb2 94-7 and anti-HAV receptor antibodies 190/4, 235/4, and 263/6 to GL37 cells was confirmed by immunofluorescence staining (Fig. 2). Neither mAb2 94-2 nor NMS bound to GL37 cells.

We performed competitive inhibition ELISA to confirm that mAb2 94-7 and anti-HAV receptor antibody shared the same HAV cellular receptor. Anti-HAV receptor antibody 235/4 and mAb2 94-7 competed with the HRPO anti-HAV receptor 190/4C for binding to HAV cellular receptors. Figure 3 shows that the inhibition rates were proportional to the concentrations of the competitors. At a 1:100 dilution, the inhibition rates of the positive competitor, mAb2 94-7 and 94-2, and the negative competitor were 93.2, 59.2, 20.8 and 18.5%, respectively. The inhibition rate of mAb2 94-7 was lower than that of the anti-HAV-receptor antibody 235/4, but higher than that of the mAb2 94-2 or the negative competitor.

MAb2-mediated protection of GL37 cells from HAV infection

The rates of mAb2 94-7-mediated blocking of GL37 cell infection with HAV in $TCID_{50}$-infectivity assays were 65.8, 54.1, and 86.0% for genotypes IA, IB, and IIIB, respectively. Blocking rates are expressed as dose-response curves (Fig. 4). On the other hand, mAb2 94-2 enhanced HAV propagation rather than protecting the cells.

Immunofocus assays showed that the number of immunofoci of genotype IIIB strain KRM003 was reduced by 83.3% in the presence of mAb2 94-7 diluted 1:40. The results were similar for genotypes IA and IB, which reduced the number of immunofoci by 71.1 and 77.8%, respectively, at the same dilution. In contrast, mAb2 94-2 did not reduce the numbers of immunofoci.

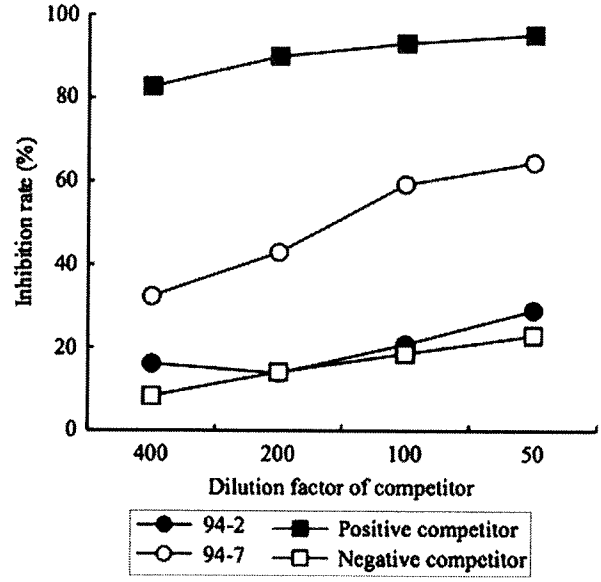

Fig. 3 Binding of mAb2s to GL37 cells in competition with anti-HAV receptor antibody. Positive competitor (*filled square*) and mAb2 94-7 (*open circle*) interfered with HRPO anti-receptor antibody 190/C binding to GL37 cells by recognizing the common HAV receptors of GL37 cells. The competitive inhibition rates of mAb2 94-2 (*filled circle*) and of negative competitor (*open square*) were equally low

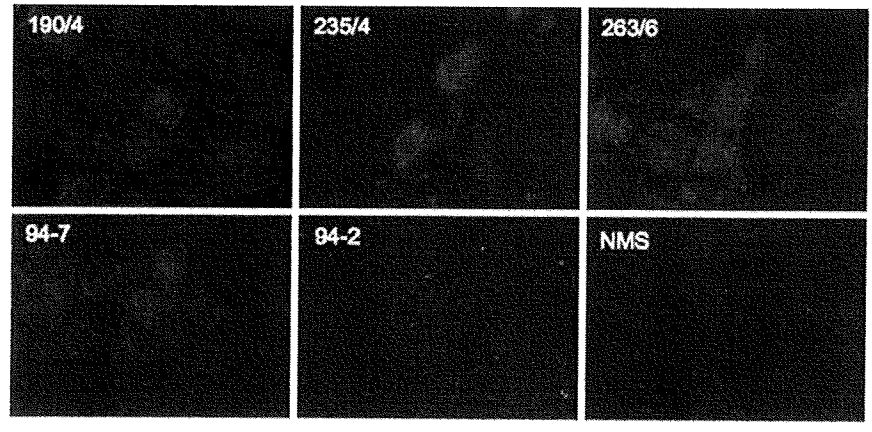

Fig. 2 Binding of mAb2s to GL37 cells. MAb2 94-7 and anti-receptor antibodies (190/4, 235/4 and 263/6) bound to GL37 cells were detected by immunofluorescence assay. MAb2 94-2 and normal mouse serum (*NMS*) were undetectable

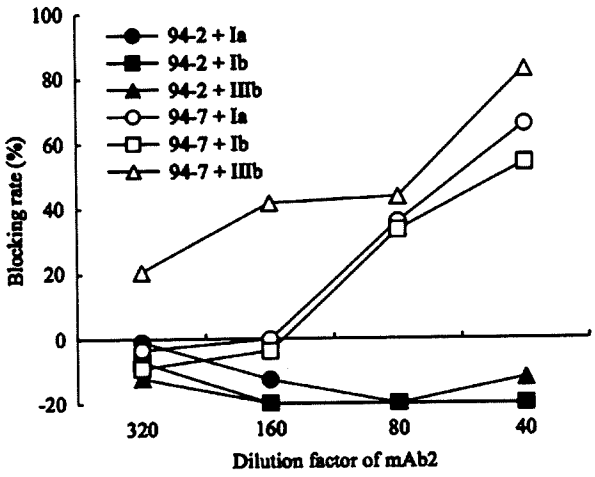

Fig. 4 MAb2-mediated protection of GL37 cells from HAV infection determined by $TCID_{50}$-infectivity assay. GL37 cells were protected from HAV infection by mAb2 94-7, but not by mAb2 94-2. Combinations of mAb2 and HAV genotypes: *filled circle* 94-2 and IA; *filled square* 94-2 and IB; *filled triangle*, 94-2 and IIIB; *open circle*, 94-7 and IA; *open square*, 94-7 and IB; *open triangle*, 94-7 and IIIB

Discussion

We generated the anti-idiotypic antibodies mAb2 94-2 and 94-7 by immunizing a mouse with anti-HAV neutralizing antibody KF94. The mAb2s were specific for the parental anti-HAV antibody KF94 and did not cross-react. They inhibited the binding of KF94 to HAV (Fig. 1a), and inactivated HAV competitively inhibited the binding of KF94 to the mAb2s (Fig. 1b), suggesting that the mAb2s and HAV bound to the paratope of KF94. Each mAb2 recognized different idiotopes within the paratope and could bind to KF94 as a surrogate of HAV.

These data indicated that the mAb2s mimic an HAV neutralization site that is complementary to the paratope of KF94. However, mimicry of the neutralization site by mAb2s might be incomplete, because the mAb2s inhibited KF94-HAV binding by only about 60%.

The characteristics of the mAb2s differed with respect to their affinity for the HAV-susceptible cell line GL37. The mAb2 94-2 neither bound to GL37 cells (Fig. 2) nor inhibited HAV infection (Fig. 4). The mAb2 94-2 seemed to have mimicked a portion of the antibody-binding site in the HAV neutralization site. The mimicked antibody-binding site interfered with KF94-HAV binding but did not influence virus-cellular receptor interaction.

On the other hand, mAb2 94-7 competed with the anti-HAV-cellular-receptor antibodies for binding to GL37 cells (Figs. 2, 3). The binding of mAb2 94-7 to GL37 cells partially blocked HAV infection (Fig. 4). We postulate that the mAb2 94-7 mimicked the part of the neutralization site that contains functional antibody-binding and cellular-receptor-binding sites.

The speculation that mimicry of the HAV neutralization site by mAb2 would be incomplete also explains why mAb2 94-7 could not totally block HAV infection. MAb2 94-7 was capable of blocking the infectivity of different genotypic strains (Fig. 4) and thus seems to mimic a common receptor-binding site among genotypes IA, IB and IIIB. Thus, these genotype strains might infect GL37 cells via a common receptor. Among the three genotypes, the rate at which mAb2 94-7 blocked infection was highest against strain IIIB (Fig. 4), which might be because KF94 was prepared from a patient infected with HAV strain IIIB.

Anti-idiotypic antibodies induced by immunization with an anti-Sindbis-virus neutralizing antibody competed with the virus for cellular receptors [22]. This suggested that a crucial receptor-binding site exposed on the viral surface is recognized by Ab1. We also speculate that the HAV receptor-binding site is exposed on the viral surface, because mAb2 94-7 competed with HAV for cellular receptors. Furthermore, the syngeneic mAb2 94-2 mimics part of the antibody-binding site. These data indicate that the antibody- and receptor-binding sites mimicked by mAb2s are exposed on the viral surface and are in close vicinity or overlap, thus comprising an epitope that could induce KF94.

Unlike other members of the family *Picornaviridae* [3], very little is understood about HAV neutralization sites and relationships between antibody- and receptor-binding sites. However, all published data support the notion that major and minor immunodominant neutralization sites exist on HAV virions and empty capsids [17, 19]. The immunodominant neutralization sites of native particles appear to be conformational and generally differ from those of denatured particles or isolated HAV structural proteins. Antibodies elicited by immunization with native or formalin-inactivated virus have broad neutralizing activity against different strains [20]. In contrast, the development of neutralizing antibodies in response to individual structural proteins, synthetic peptides, or expressed uncleaved precursors and polypeptides is problematic [7, 10, 15]. Thus, analysis of HAV neutralization sites using such probes is not simple. Analysis of HAV neutralization sites has mainly depended on the use of neutralization-escape mutants generated by serial passage of the virus in cultured cells in the presence of neutralizing mouse monoclonal antibodies [17, 19]. Although HAV strains isolated from various parts of the world belong to a single serotype [18], neutralization-escape mutants can be produced experimentally. These mutants are expected to possess a replicative advantage and survive more efficiently than wild-type virus as a result of the arrangement of surface neutralizing antibody-binding sites. However,

their in vivo replication is restricted compared with wild-type virus, although they appear to be equally stable in vitro [14]. Furthermore, although the rate of substitution throughout the HAV genome is high, most of the mutations are silent [6]. Presumably, alterations in neutralizing-antibody-binding sites, such as in neutralization-escape mutants, might arise during natural infection. However, constraints probably prevent this from occurring in nature. Our results indicated that one such constraint depends on the relationship between antibody- and receptor-binding sites on the viral surface. These sites might be closely located or overlap within an immunodominant neutralization site. Therefore, mutations on the antibody-binding site might result in deterioration of the receptor-binding site. Such deterioration would consequently reduce the affinity of the HAV receptor-binding site, which would result in neutralization escape mutants being unable to replicate to significant levels in vivo. Alterations in antibody-binding sites of poliovirus are thought to be responsible for the affinity of the receptor-binding site [8]. The relationship between the antibody- and receptor-binding sites shown by mAb2s might partly explain why only one serotype of HAV exists.

To our knowledge, we are the first to analyze the HAV surface using anti-Ids to mimic receptor- and antibody-binding sites within HAV neutralizing sites. Our anti-Ids could not completely mimic the viral surface structure, but they remained functionally intact. Anti-Id antibodies may be considered obsolete, but they nevertheless represent an effective tool with which to structurally analyze the viral surface.

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