Adherence of GAS isolates to mammalian cells

To compare the ability of these GAS isolates to adhere to mammalian cells, we utilized L cells, a mouse fibroblast cell line. The adhesion of these GAS isolates to L cells showed a significant inverse association with their virulence in mice ($P = 0.0025$) (Fig. 2a). During the period of coculture with L cells (4 h), all isolates except ST10 showed essentially the same growth behavior (data not shown). When L cells were treated with the least virulent isolate, SF10, but not the most virulent isolate, ST1, GAS bacterial cells were observed on the surface of L cells. However, there was no difference in appearance between L cells incubated with SF10 and ST1, indicating that the difference in adhesion ability between these two isolates was not due to physical modification of L cells, such as disappearance from the assay system (Fig. 2b). The lower adherence of ST1 compared with SF10 was also observed when these isolates were incubated with the human-derived cell lines HEp-2 and HeLa (Fig. 2c), although the difference in adhesion between ST1 and SF10 was relatively small. These results indicated that high-virulence GAS isolates have lower ability to adhere to mammalian cells than low-virulence isolates.

To confirm that high-virulence GAS isolates have lower ability to adhere to mammalian cells *in vivo*, these isolates were injected into mice (intraperitonially) and recovered from the peritoneal cavity 30 min later. When we plotted the number of bacteria recovered for each isolate against its corresponding virulence, we found that GAS virulence in mice was correlated positively with GAS recovery from the peritoneal cavity ($P < 0.00001$; Fig. 3). As recovery was performed 30 min after injection, it is highly unlikely that the observed differences in number of bacteria recovered were due to bacterial growth. We previously reported that phagocytic cells (neutrophils) were induced by intraperitonial administration of GAS in mice (Miyoshi-Akiyama *et al.*, 2005). This induction required at least 3 h and maximized 12 h after GAS inoculation. Thus, the contribution of phagocytic killing of bacterial cells to the number of GAS recovered from peritoneal cavity was also minimal. These

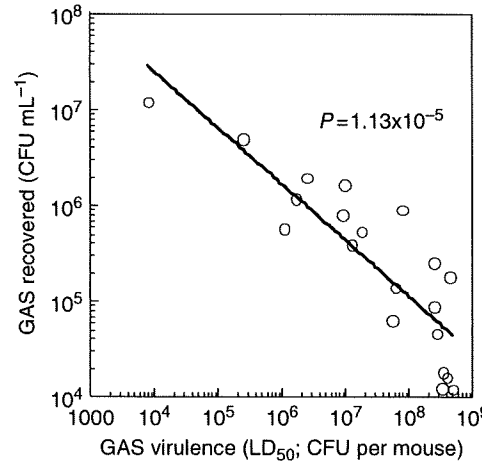

Fig. 3. Correlation of virulence with GAS recovery from the peritoneal cavity after intraperitonial injection into mice. GAS were recovered from the peritoneal cavities of mice 30 min after intraperitonial injection of the GAS isolates suspended in PBS, as described in Materials and methods. The number of GAS recovered (CFU mL^{-1}) was plotted against virulence in mice (LD$_{50}$; CFU per mouse). The correlation was analyzed by Kendall's rank correlation analysis ($P = 1.13 \times 10^{-5}$).

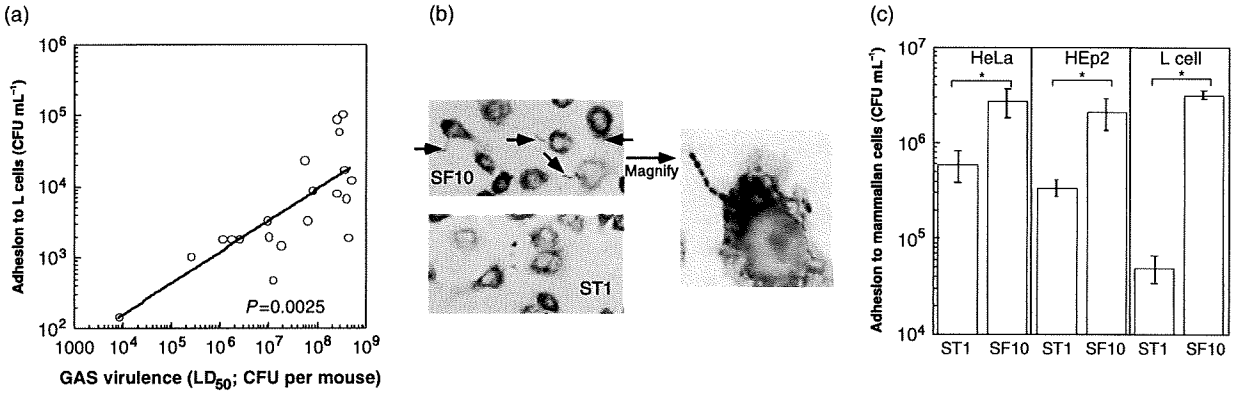

Fig. 2. Adhesion of the GAS isolates to mammalian cells. (a) Correlation of the lower adhesion of the isolates with virulence in the mouse model. Adhesion of the GAS isolates to L cells was analyzed as described in Materials and methods, and the numbers of adhering GAS cells (CFU mL^{-1}) were plotted against the mouse virulence (LD$_{50}$; CFU per mouse). During the 4-h analysis period, all isolates except ST10 showed essentially the same growth behavior (data not shown). Correlations were analyzed by Kendall's rank correlation analysis ($P = 0.0025$). (b) L cells were incubated with SF10 (the least virulent isolate) or ST1 (the most virulent isolate) as described in Materials and methods. After washing with PBS, Giemsa staining was performed. Bacterial cells seen in L cells are marked with arrows in the figure. (c) The indicated cell lines were incubated with SF10 or ST1 to compare differences in GAS adhesion. The data are presented as means ± SE. Statistical significance of the difference of ability to adhere cell lines between ST1 and SF10 were analyzed by Student's *t*-test (*$P < 0.01$).

results indicated that GAS isolates that showed higher recovery rates were trapped less frequently in the mouse peritoneal cavity than GAS isolates that showed lower recoveries.

GAS invasion into the bloodstream in mice

Next we investigated whether there was a correlation between the invasiveness of GAS isolates into the bloodstream and lethality in mice. The GAS isolates were injected into mice (intraperitonially), and blood samples were obtained by cardiac puncture 3 h later. We found that the appearance of GAS in the bloodstream after injection into the peritoneal cavity was significantly correlated with virulence ($P < 0.00001$, Fig. 4), suggesting that GAS isolates not trapped on host cells due to their lower ability to attach appear in the bloodstream more rapidly than GAS isolates that attach more efficiently to the host cells. Our results agree with previous findings, showing that GAS isolated from the blood shows lower adherence than GAS isolated from the throat or skin (Molinari & Chhatwal, 1998).

Comparison of molecular features of the GAS isolates

As the low adhesion of GAS to L cells was correlated with high virulence in mice, the lower adherence reflects the ability of GAS to spread in the body. To identify possible candidates that may affect the ability to adhere to host cells, we examined the expression patterns of molecules reported as 'adhesins' of GAS.

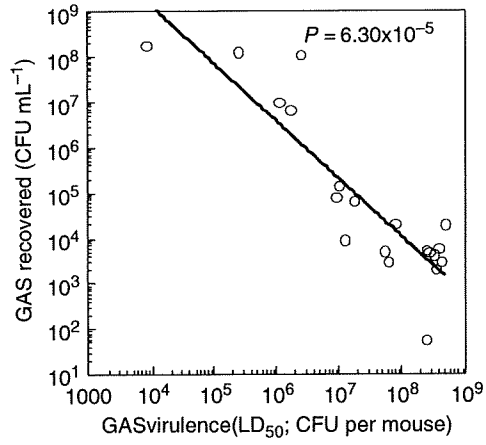

Fig. 4. Correlation of virulence with GAS recovery in heart blood after intraperitonial injection into mice. GAS were recovered by puncture of the heart 3 h after intraperitonial injection of the GAS isolates suspended in PBS as described in Materials and methods. The number of GAS recovered (CFU mL^{-1}) was plotted against virulence in mice (LD_{50}; CFU per mouse). The correlation was analyzed by Kendall's rank correlation analysis ($P = 6.30 \times 10^{-5}$).

GAS with mutations in the M protein and protein F genes showed markedly decreased host cell adhesion (Okada *et al.*, 1994). To compare the expression level of M protein among the GAS isolates, an antibody against the constant region of M6 protein was used for Western blotting analysis (Fig. 5a). It is not appropriate to compare the M protein expression levels among GAS isolates carrying different types of *emm*, such as *emm4*, *emm3*, and *emm12*. ST1 to ST7, however, have similar genetic backgrounds, as shown on PFGE analysis (Fig. 1), although these isolates showed marked differences in adhesion to L cells and virulence in mice. There were no significant differences in M protein expression level among these seven isolates, suggesting that M protein is not responsible for the difference in the ability of GAS isolates to adhere to host cells.

Because the hyaluronic capsule content of GAS isolates has been reported to act as an adherence factor via binding with CD44 (Schrager *et al.*, 1998; Darmstadt *et al.*, 2000), to prevent phagocytic killing in co-operation with M protein, we assessed the hyaluronic acid capsule content in each GAS isolate. There was no correlation between the hyaluronic acid content of each GAS isolate and its virulence (Fig. 5b) ($P > 0.9$). Although ST1, which has a mucoid phenotype, showed the highest hyaluronic acid content, other isolates showed similar hyaluronic acid levels. These observations suggest that differences in hyaluronic acid content among the isolates cannot explain the observed differences in their virulence.

We also examined the profile of fibronectin-binding proteins produced by the GAS isolates. GAS produces many factors that bind fibronectin (Bisno *et al.*, 2003), one of the major extracellular matrix proteins. Because distribution of the fibronectin-binding proteins in GAS depends to some extent on their *emm* type (Kratovac *et al.*, 2007) and the *emm* types of the isolates used in this study differ from each other, we performed far Western blotting, using fibronectin as a probe, to distinguish among them (Fig. 5c). Several proteins were detected in each isolate, and there were similarities among the profiles of the different isolates. By PCR, we confirmed that M1 and M3 type GAS, corresponding to the ST1, ST3-7, and SF1 isolates, do not carry the protein F gene (data not shown). Probing with fibronectin, however, detected proteins of around 110–130 kDa in ST3 to ST6 and 70–100 kDa in ST1 and ST2. Although proteins from the GAS isolates carrying *emm* other than *emm3* (and *emm89*) showed reactivity with fibronectin, their expression level was not correlated with virulence or adhesion, suggesting that fibronectin-reactive proteins of GAS are not responsible for the differences in their ability to adhere to host cells.

Discussion

Our results are somewhat surprising, as GAS virulence was negatively correlated with the ability to adhere to

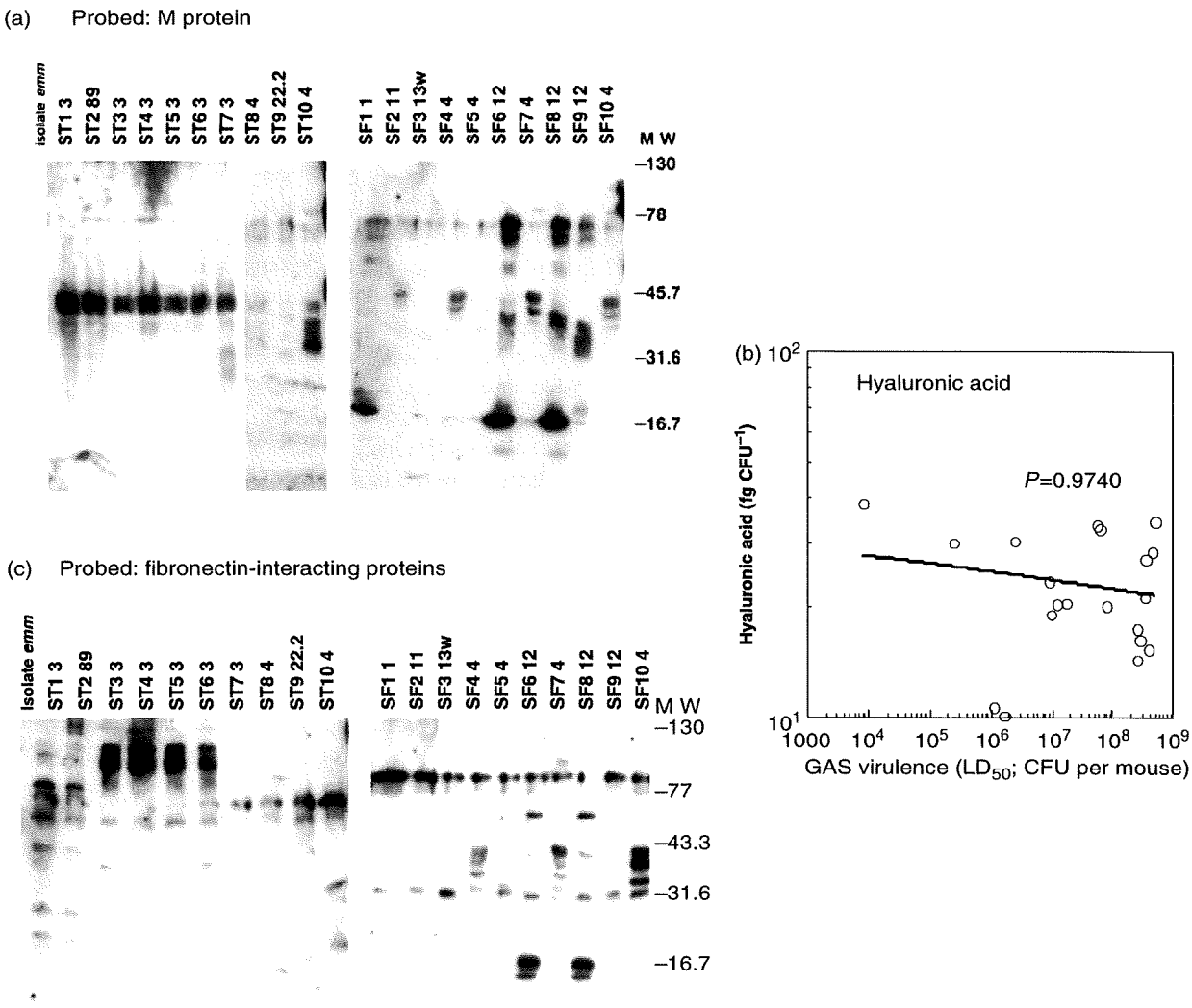

Fig. 5. Analyses of traits related to mammalian cell adhesion of the GAS isolates. (a) M protein expression level in the GAS isolates. GAS lysates, prepared as described in Materials and methods, were blotted and probed with antiserum against the constant region of the M6 protein. Because of differences in M-types among the isolates, the molecular weights of the M proteins are not the same. (b) Hyaluronic acid contents of the GAS isolates. Hyaluronic acid was quantified using Stains-All as described in Materials and methods, and the values were plotted against the virulence of the GAS isolates in mice. The correlation was analyzed by Kendall's rank correlation analysis ($P = 0.9740$). (c) Analysis of fibronectin-interacting proteins in the GAS isolates. GAS lysates prepared as described in Materials and methods were blotted and probed with fibronectin. Binding of fibronectin to the GAS proteins was detected with antifibronectin antibody.

mammalian cells. Assessments of the adhesion of GAS isolates from STSS and other GAS infectious diseases, such as superficial disease, showed only slight differences between them (Bennett-Wood *et al.*, 1998). In the present study, however, we found 10–100-fold differences between isolates using L cells as mammalian host cells. Among the mammalian cells tested, L cells showed the highest differences in the adherence ability of GAS isolates, suggesting that the discrepancies between our results and those of previous studies may be due to differences in the cell lines used. The inverse correlation between adherence to L cells and virulence in mice was maintained in additional 32 GAS clinical isolates tested (data not shown). Because higher amounts of GAS isolates showing lower adherence to host cells were recovered from the peritoneal cavities of mice after intraperitonial injection, compared with the recovery of more highly adherent GAS isolates, the *in vitro* assay we employed reflected *in vivo* conditions, at least in this mouse model.

Several previous studies have shown that the genetic disruption of GAS molecules that affect bacterial adherence to mammalian cells attenuated the virulence in mouse models (Terao *et al.*, 2001, 2002b; Okamoto *et al.*, 2004). These findings, however, differed from our results and from those of a previous study regarding protein F1 (Nyberg *et al.*,

2004). This discrepancy may be due, at least in part, to the route of GAS injection; some of the former studies employed subcutaneous and intranasal injections, while the latter studies and ours employed the intraperitonial route. Intraperitoneal injection makes it possible for GAS to gain direct access to normally sterile sites within the body without a previous colonization step. In many patients with STSS, GAS enters through an injury or wound. In such cases, GAS isolates that do not adhere tightly to the host cells could spread more rapidly in the body than those that become trapped at sites by adhesion to host cells. Intraperitoneal injection of GAS may mimic this process. Adhesion is clearly necessary for other types of GAS infectious diseases, such as pharyngitis. In these individuals, GAS attaches to host cells at other locations, such as the throat, and may then colonize the site to begin the infection. The transcriptome of GAS has been reported to change dramatically, allowing GAS to spread after entry into the sterile part of the body (Graham *et al.*, 2006). Our findings, based on these phenotypic analyses, support these previous findings

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Adhesion of M1, M3, M4, and M12 GAS isolates to mammalian cells.

Table S1. The reproducibility of LD_{50} values of GAS isolates in a mouse intraperitonial infection model during 1999–2006.

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