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NOTES

Effect of Neutralizing Sera on Factor X-Mediated Adenovirus Serotype 5 Gene Transfer$^{\triangledown}$

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The deployment of adenovirus serotype 5 (Ad5)-based vectors is hampered by preexisting immunity. When such vectors are delivered intravenously, hepatocyte transduction is mediated by the hexon-coagulation factor X (FX) interaction. Here, we demonstrate that human sera efficiently block FX-mediated cellular binding and transduction of Ad5-based vectors in vitro. Neutralizing activity correlated well with the ability to inhibit Ad5-mediated liver transduction, suggesting that prescreening patient sera in this manner accurately predicts the efficacy of Ad5-based gene therapies. Neutralization in vitro can be partially bypassed by pseudotyping with Ad45 fiber protein, indicating that a proportion of neutralizing antibodies are directed against the Ad5 fiber.

Adenoviruses, particularly those based on adenovirus serotype 5 (Ad5), are used for a variety of gene delivery applications both in vitro and in vivo, since they efficiently transduce a wide range of cell types. In vitro, primary cell tethering involves the Ad5 fiber knob domain engaging coxsackievirus and adenovirus receptors (CAR) (3). Once bound, the flexible fiber protein bends, allowing a secondary, uptake-stimulating interaction to occur between recruited $\alpha_v\beta_3/\alpha_v\beta_5$ integrins and RGD motifs in the penton base (28). When introduced systemically, the transduction profile of Ad5, characterized by extensive hepatocyte transduction, does not correlate with expression of CAR, which is limited to the tight junctions (5). Furthermore, the introduction of mutations within the fiber knob domain that abrogate CAR binding and infectivity in vitro has no discernible effect on Ad5 biodistribution and transduction profiles in vivo following intravenous delivery (1, 10). Together, these observations suggested that Ad5-mediated transduction of hepatocytes operate via a CAR-independent pathway. Recently, several studies (7, 11, 12, 16, 17, 24, 26, 27) have suggested a fundamental involvement of host proteins, notably coagulation factors, in dictating adenoviral infectivity in vivo. Pivotal to hepatic transduction is the interaction between the Ad5 hexon protein and the coagulation factor X (FX) (7, 24, 26), which serves to "bridge" the Ad5-FX complex to alternative receptors, which are expressed abundantly within the liver. Hepatocyte transduction can be abolished by using warfarin (7, 12, 16, 27) to prevent the gamma carboxylation of glutamic acid residues in the N-terminal γ-carboxyglutamic acid (Gla) domain of FX; the snake venom-derived protein X-bp (2), which binds the Gla domain of FX, inhibiting its interaction with hexon (26); or hexon chimeric or mutated Ad5 vectors to abrogate FX binding (7, 24, 26).

Forty-five to fifty percent of individuals in the United States possess high levels of neutralizing antibodies (NAbs) against the Ad5 vector, with even higher seroprevalence rates observed among Asian and African populations (21). NAbs associate with the capsid, preventing transduction of the target cell (19, 20) and limiting its efficacy for gene transfer in vivo (8), while serving to prime a robust immunological response against the vector (4). Existing assays for the detection of NAbs are based on inhibiting CAR-mediated uptake (6, 9, 13, 19), a pathway that appears to be redundant for intravenous targeting (12, 26). Since several previous studies have implicated the hexon protein as the major site of immunogenicity on the Ad5 capsid (14, 15, 21, 30), we reasoned that it would be important to assess the effects of neutralizing sera on cellular uptake mediated via the hexon-FX pathway. We considered that examining the effects of sera on FX-mediated uptake would better predict the clinical efficacy of Ad5-based vectors when delivered systemically and would show improved sensitivity compared with that of existing assays.

To examine the neutralizing capacity of patient sera on FX-mediated Ad5 uptake, we examined the capacity of Ad5 to bind and infect the human hepatoma cell line (HepG2) in

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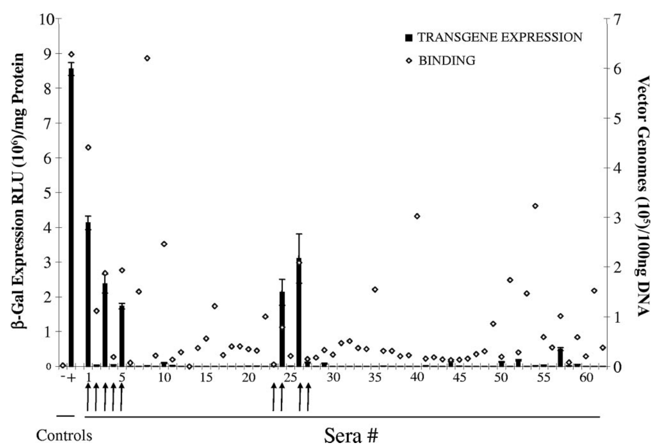

FIG. 1. Effect of neutralizing sera on FX-mediated Ad5 cell binding and transduction. HepG2 cells were infected with 1,000 vp/cell of AdKO1 in the presence of 1 IU/ml of FX and 2.5% sera from patients previously screened for anti-Ad NAbs and stained for expression of the β-Gal reporter gene 48 h postinfection. Controls were cells only or virus in the presence or absence of FX (in the absence of serum). RLU, relative light units. Quantification of cell transduction (bars) and binding (diamonds) of Ad5 in the presence of neutralizing sera was performed. A binding assay was performed as described previously (11), quantifying the binding of Ad5 to HepG2 cells following 1 h of incubation at 4°C. Vector genomes were detected by quantitative PCR, using the LacZ-specific primers 5′-ATCTGACCACCAGCGAAATG G-3′ and 5′-CATCAGCAGGTGTATCTGCCG-3′.

vitro, in the presence of both physiological concentrations of FX (8 μg/ml) and 2.5% neutralizing sera. Since liver infectivity in vivo is not mediated via the CAR pathway, we utilized the CAR-binding ablated vector AdKO1 (18) for in vitro studies to best replicate the in vivo setting. Forty-eight hours postinfection, reporter transgene activity (β-galactosidase [β-Gal]) was quantified (Fig. 1). All sera tested had previously been evaluated for the presence of NAbs by using existing assays based on the infectivity of Ad5 in A549 cells, with sera 1, 2, 3, 4, 5, 23, 24, 26, and 27 all scoring negative for neutralization (Table 1). The majority of sera that had previously tested positive for neutralizing activity using the CAR-based infectivity assay in A549 cells also efficiently neutralized FX-mediated Ad5 transduction. However, four of the sera (sera 2, 4, 23, and 27) which had previously been scored negative for neutralization by the CAR-based infectivity assays in A549 cells neutralized efficiently in the FX-based assay. To examine whether this neutralizing capacity correlated with a decreased level of binding of virions to cell surfaces, we performed cell tethering studies at 4°C as described previously (Fig. 1) (11). For the majority of sera tested, low levels of transduction correlated well with decreased levels of cell binding. Several sera (for example, sera 8, 10, 40, and 54), however, demonstrated considerable levels of cell association in the absence of transduction, indicating that neutralization is likely to have occurred at a post-cell binding step, as has been observed previously for some NAbs (23).

To further evaluate the sensitivity of the FX-mediated neutralization assay for detecting NAbs, we performed assays in the presence of FX and limiting dilutions of sera (Fig. 2) that neutralize in both FX- and CAR-based assays (sera 14 and 16), that neutralize poorly in both assays (sera 1 and 26), or that neutralize in the FX- but not the CAR-based assay (sera 2 and

TABLE 1. Effect of neutralizing sera on CAR-mediated Ad5 transduction[a]

Sera no.	% Sera required to inhibit Luc expression by >90% in A549 cells	NAb status
1	**>25**	**−**
2	**>25**	**−**
3	**>25**	**−**
4	**>25**	**−**
5	**>25**	**−**
6	6.7	+
7	1.6	+
8	7.5	+
9	2.3	+
10	2.8	+
11	5.7	+
12	2.3	+
13	1.9	+
14	2.1	+
15	5.8	+
16	1.6	+
17	9.0	+
18	9.1	+
19	7.3	+
20	2.5	+
21	4.3	+
22	2.4	+
23	**>25**	**−**
24	**>25**	**−**
25	1.6	+
26	**>25**	**−**
27	**>25**	**−**
28	4.2	+
29	3.0	+
30	1.6	+
31	5.4	+
32	5.4	+
33	2.6	+
34	5.1	+
35	5.6	+
36	1.6	+
37	7.5	+
38	1.6	+
39	1.6	+
40	1.6	+
41	4.2	+
42	1.6	+
43	1.6	+
44	3.0	+
45	4.7	+
46	2.8	+
47	1.9	+
48	3.4	+
49	1.8	+
50	3.8	+
51	3.9	+
52	3.0	+
53	1.6	+
54	1.6	+
55	5.2	+
56	1.6	+
57	6.1	+
58	1.6	+
59	3.0	+
60	1.6	+
61	8.2	+
62	4.0	+

[a] A549 cells were transduced with Ad5 in the presence of sera derived from 62 individuals. The percentage of sera required to inhibit luciferase (Luc) by >90% was gauged. Sera which failed to inhibit >90% of luciferase expression in 25% sera (the highest concentration of sera tested) were considered negative for neutralizing antibodies (boldface).

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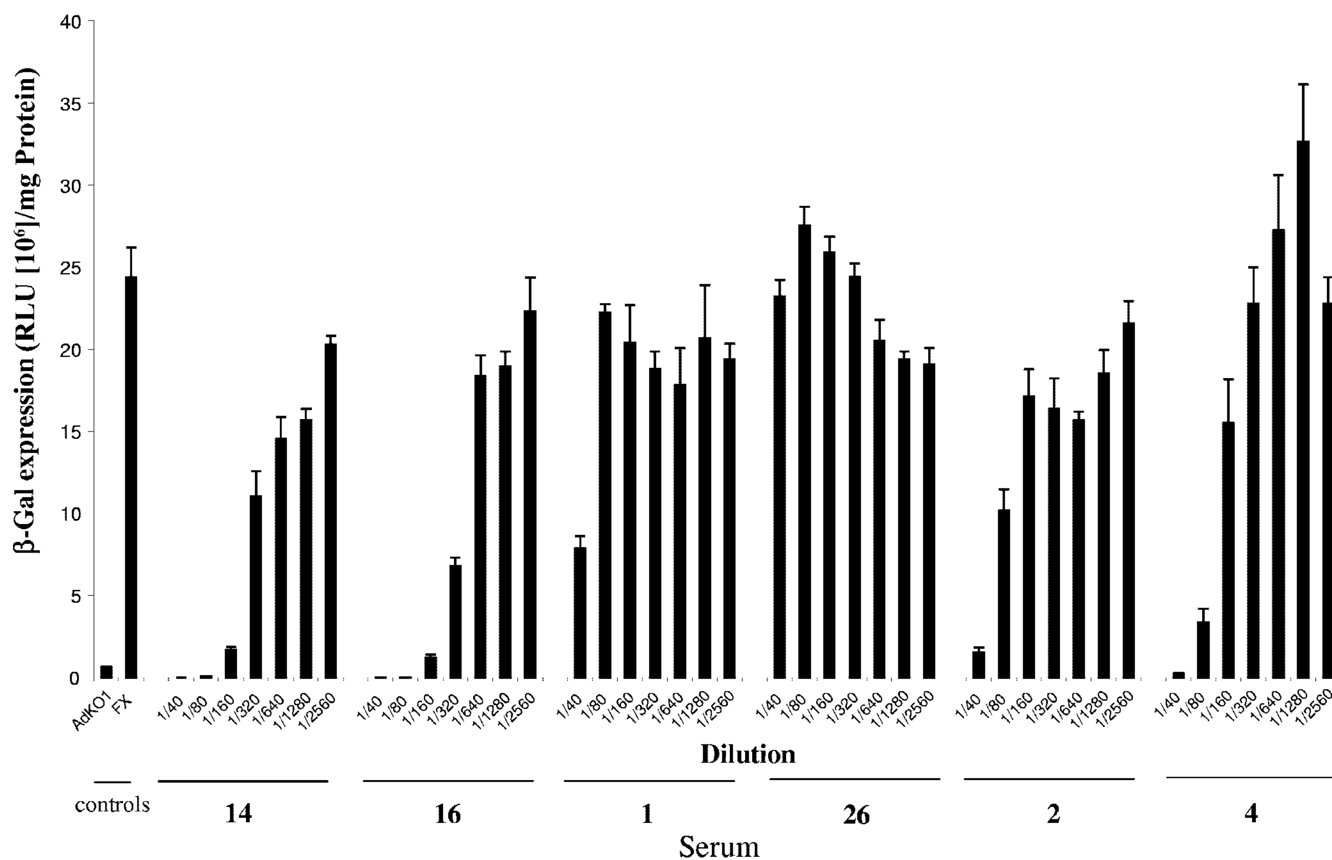

FIG. 2. Effect of serum dilution on FX-mediated cellular transduction. HepG2 cells were infected with AdKO1 in the presence of 1 IU/ml of FX and in the presence of doubling dilutions of selected neutralizing sera. Expression of the β-Gal reporter was detected 48 h postinfection. RLU, relative light units.

4). Serum 1 showed detectable but weak neutralization (30% at a 2.5% dilution), while serum 26 showed no neutralization at any dilution. While strong neutralization of transgene expression was noted for sera 14 and 16, sera 2 and 4 showed more intermediate neutralization of transduction. These data suggest that neutralization appears more pronounced via the FX-based assay and that this assay might be more relevant for gauging the efficacy of Ad-based vectors designed for application via the systemic route.

To determine the effects of NAbs on transduction of hepatocytes in vivo following systemic delivery, mice were given Ad5 in the presence or absence of selected neutralizing sera. In order to maintain a serum/virus ratio consistent with the in vitro experiments and to avoid injecting large volumes of sera, we elected to administer 1×10^9 viral particles (vp) of Ad5 with and without 125 μl undiluted neutralizing sera. To facilitate transduction of hepatocytes at such low virus doses, mice were transiently depleted of macrophages by pretreatment with 200 μl of liposomal clodronate 24 h prior to the administration of the virus-serum (22). In the absence of neutralizing sera, Ad5 mediated high levels of transduction in the liver (Fig. 3). In the presence of serum 14 (a highly neutralizing serum in vitro), transduction in the liver was completely ablated, indicating that efficient viral neutralization had occurred, preventing infection of hepatocytes. Sera with intermediate (serum 27) or minimal (serum 26) neutralizing activity in vitro demonstrated levels of transduction that were intermediate between the two extremes, indicating a partial neutralization of the vector. These data demonstrate the enhanced sensitivity of the in vitro FX-based neutralization assay in identifying serum samples with potential anti-Ad5 activity relevant to systemic in vivo gene therapy applications.

To evaluate whether pseudotyping the Ad5 vector with fibers from an alternative and less-immunogenic serotype (Ad45) might represent an effective strategy for bypassing NAbs, neutralization experiments were performed using the pseudotyped Ad vector Ad5/f45 (Fig. 4). Ad45 has been shown previously to exhibit greatly reduced seroprevalence compared with that of Ad5 (25), while the pseudotyped vector Ad5/f45 demonstrated substantial FX-mediated enhanced cell binding and transduction (11). Surprisingly, we found that the pseudotyped vector was able to mediate significant transgene expression in the presence of a number of neutralizing sera, suggesting that a significant proportion of NAbs appeared to be directed against the Ad5 fiber protein. Of the 62 sera tested, 57 sera (91.9%) mediated >90% inhibition of transduction for Ad5, whereas only 35 sera (56.5%) mediated >90% inhibition of transduction for Ad5/f45 at the same dilution, suggesting that a significant proportion of NAbs in human sera are directed against the fiber protein and that antibody evasion might be achieved (at least partially) by pseudotyping Ad5 with fibers from less-immunogenic serotypes, rather than complete

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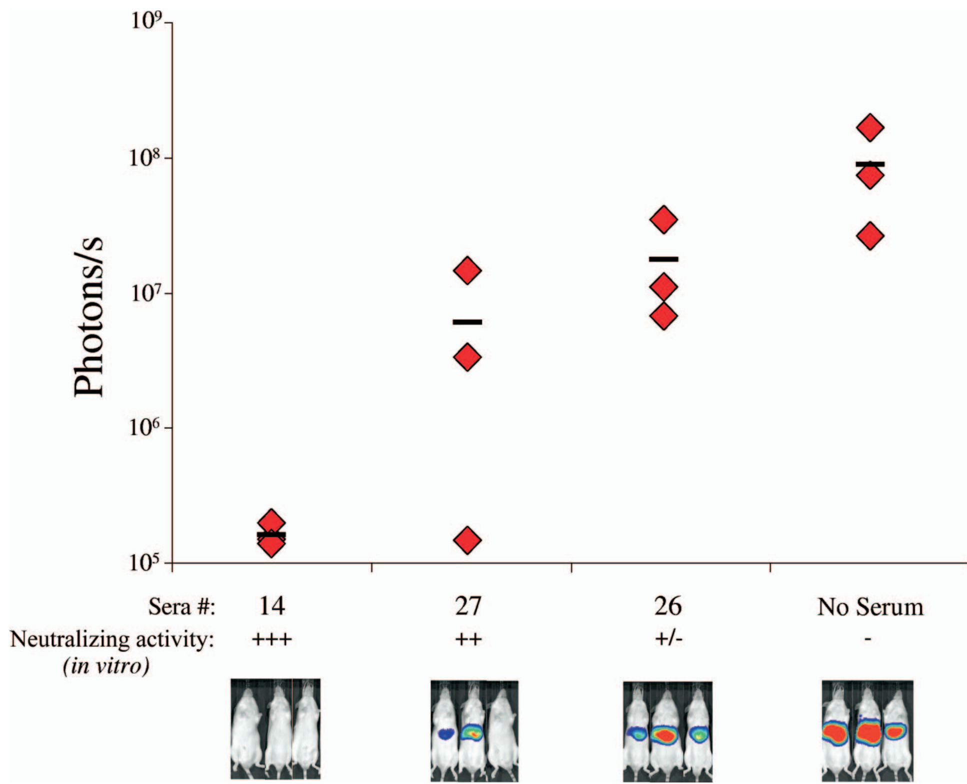

FIG. 3. Effect of neutralizing sera on Ad-mediated transduction of hepatocytes in vivo. Outbred MF-1 mice were transiently depleted of macrophages by the administration of 200 μl of liposomal clodronate 24 h prior to the intravenous administration of 1×10^9 vp of Ad5 in the presence or absence of 125 μl of neutralizing sera from selected individuals. At 48 h, an intraperitoneal injection of luciferin was followed by in vivo bioimaging with a Xenogen IVIS-50 cooled charge-coupled-device camera. Luciferase expression was quantitated as photon flux from the upper abdomen (diamonds).

serotype switching. Antibodies directed against the Ad5 fiber protein may neutralize infectivity either by preventing cellular attachment by interfering with the fiber knob-CAR receptor interaction or by inducing the aggregation of virions, thereby providing an extracellular means of neutralization (29). Pseudotyping of Ad5 with the fiber protein from the less-seroprevalent Ad45 may prevent neutralization by one or both of these mechanisms.

Taken together, these data suggest that the neutralizing activity of human sera can be accurately gauged via FX-mediated

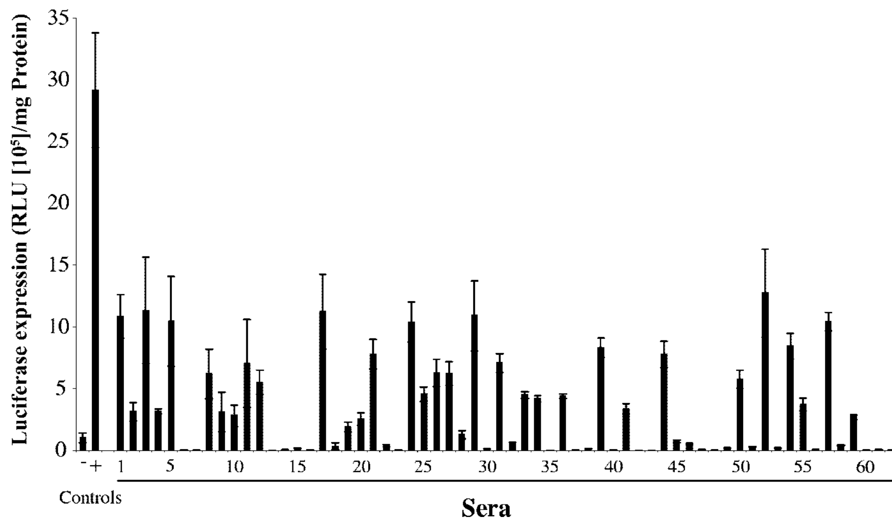

FIG. 4. Effect of neutralizing sera on FX-mediated cell infectivity of fiber-pseudotyped Ad vectors. HepG2 cells were infected with 1,000 vp/ml of Ad5/f45 in the presence of 1 IU/ml of FX and 2.5% neutralizing sera. Reporter gene activity was quantified 48 h postinfection.

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uptake. The degree of neutralizing activity, as established by an in vitro assay, appears to correlate with the efficiency of hepatocyte transduction in vivo in the presence of neutralizing sera, a trait that ultimately will be important when considering the systemic application of Ad5-based vectors clinically. Furthermore, we note that the pseudotyping of Ad5 vectors with the fiber protein from less-immunogenic serotypes resulted in a markedly decreased ability of NAbs to limit FX-mediated hepatocyte transduction, suggesting that fiber pseudotyping may, at least partially, represent a strategy to overcome vector neutralization following systemic administration.

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