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Variability of Human Systemic Humoral Immune Responses to Adenovirus Gene Transfer Vectors Administered to Different Organs

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Administration of adenovirus (Ad) vectors to immunologically naive experimental animals almost invariably results in the induction of systemic anti-Ad neutralizing antibodies. To determine if the human systemic humoral host responses to Ad vectors follow a similar pattern, we evaluated the systemic (serum) anti-Ad serotype 5 (Ad5) neutralizing antibodies in humans after administration of first generation ($E1^-$ $E3^-$) Ad5-based gene transfer vectors to different hosts. Ad_{GV}CFTR.10 (carrying the normal human cystic fibrosis [CF] transmembrane regulator cDNA) was sprayed (8×10^7 to 2×10^{10} particle units [PU]) repetitively (every 3 months or every 2 weeks) to the airway epithelium of 15 individuals with CF. Ad_{GV}CD.10 (carrying the *Escherichia coli* cytosine deaminase gene) was administered (8×10^8 to 8×10^9 PU; once a week, twice) directly to liver metastasis of five individuals with colon cancer and by the intradermal route (8×10^7 to 8×10^9 PU, single administration) to six healthy individuals. Ad_{GV}VEGF121.10 (carrying the human vascular endothelial growth factor 121 cDNA) was administered (4×10^8 to $4 \times 10^{9.5}$ PU, single administration) directly to the myocardium of 11 individuals with ischemic heart disease. Ad vector administration to the airways of individuals with CF evoked no or minimal serum neutralizing antibodies, even with repetitive administration. In contrast, intratumor administration of an Ad vector to individuals with metastatic colon cancer resulted in a robust antibody response, with anti-Ad neutralizing antibody titers of 10^2 to $>10^4$. Healthy individuals responded to single intradermal Ad vector variably, from induction of no neutralizing anti-Ad antibodies to titers of 5×10^3. Likewise, individuals with ischemic heart disease had a variable response to single intramyocardial vector administration, ranging from minimal neutralizing antibody levels to titers of 10^4. Evaluation of the data from all trials showed no correlation between the peak serum neutralizing anti-Ad response and the dose of Ad vector administered ($P > 0.1$, all comparisons). In contrast, there was a striking correlation between the peak anti-Ad5 neutralizing antibody levels evoked by vector administration and the level of preexisting anti-Ad5 antibodies ($P = 0.0001$). Thus, unlike the case for experimental animals, administration of Ad vectors to humans does not invariably evoke a systemic anti-Ad neutralizing antibody response. In humans, the extent of the response is dictated by preexisting antibody titers and modified by route of administration but is not dose dependent. Since the extent of anti-Ad neutralizing antibodies will likely modify the efficacy of administration of Ad vectors, these observations are of fundamental importance in designing human gene therapy trials and in interpreting the efficacy of Ad vector-mediated gene transfer.

Extensive studies in experimental animals have demonstrated the ability of $E1^-$ replication-deficient adenovirus (Ad) vectors to transfer and express transgenes in a variety of organs (2, 5, 8, 9, 22, 23, 25, 35, 39, 40, 42, 45, 51, 52, 55, 56, 59, 65, 67, 70, 71, 73–75, 78, 85, 89, 90, 97, 98, 100, 104, 107, 108, 110, 116, 117, 132, 134–138). In experimental animals, the administration of these vectors is almost invariably associated with the development of systemic neutralizing antibodies directed against the Ad vector (11, 25, 27, 31, 35, 44, 47–49, 51–53, 57, 58, 62, 63, 65, 66, 72, 76, 77, 80, 101, 103, 104, 108–110, 114, 118–121, 124, 127, 131, 132, 134–138). The anti-Ad neutralizing antibody response is robust in immunologically naive animals, with generation of a systemic anti-Ad neutralizing humoral response within 2 to 4 weeks, depending on the species. The intensity of systemic anti-Ad humoral immunity in experimental animals is dependent on the dose and on the route of administration of the vector (31, 108, 110, 120, 137).

Based on the ability of Ad vectors to safely mediate transfer and robust expression of transgenes in organs of experimental animals, these vectors are being evaluated in a variety of human gene transfer applications (4). In the context of the observation that administration of Ad vectors by a variety of routes to naive experimental animals rapidly evokes systemic anti-Ad neutralizing antibodies, the present study focuses on several questions regarding the administration of Ad vectors to humans: (i) does the administration of Ad vectors to humans invariably evoke systemic anti-Ad neutralizing antibodies; (ii) does the extent of the neutralizing antibody response depend on the route of administration; (iii) is the systemic anti-Ad humoral response dose dependent; and (iv) does the baseline anti-Ad antibody status of the human recipient modify the humoral response to administration of the vector? To accomplish this, we have evaluated our human experience with Ad

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TABLE 1. Characteristics of Ad vectors

Trial	Vector	Lot no.	PU/ml	PFU/ml	PU/PFU[a]	Doses used for each administration (PU)
Normal skin	Ad_{GV}CD.10	0061-0002	3.7×10^{11}	1.0×10^{10}	36	8×10^{7}–8×10^{9}
CF	Ad_{GV}CFTR.10	0056-0002	2.2×10^{11}	3.7×10^{10}	6	3×10^{7}–$3 \times 10^{8.5}$
q3mo × 3 (part A)						
Individuals 1–8						
	Ad_{GV}CFTR.10	0045-007	1.4×10^{12}	1.6×10^{11}	9	3×10^{9}–3×10^{10}
Individuals 9–14						
q15d × 4 (part B)	Ad_{GV}CFTR.10	0045-007	1.4×10^{12}	1.6×10^{11}	9	$3 \times 10^{9.5}$
Metastatic colon cancer	Ad_{GV}CD.10	0061-0002	3.7×10^{11}	1.0×10^{10}	36	8×10^{8}–8×10^{9}
Coronary artery disease	Ad_{GV}VEGF121.10	0061-0003	4.0×10^{11}	1.0×10^{10}	40	4×10^{8}–$4 \times 10^{9.5}$

[a] For convenience in discussing and graphing the data (Fig. 1 to 3), the ratios have been rounded off to the nearest 10 U, i.e., PU/PFU = 6 and 9 rounded to 10, and PU/PFU = 36 rounded to 40 (see Materials and Methods).

vectors administered to the airway epithelium of individuals with cystic fibrosis (CF), metastatic tumors in liver of individuals with colon cancer, the skin of healthy (normal) individuals, and the myocardium of individuals with coronary artery disease. The data demonstrate that humans can mount a systemic anti-Ad neutralizing antibody response following administration of these vectors but that the results are quite different than in experimental animals, with minimal responses in naive humans (i.e., those with no detectable preexisting anti-Ad neutralizing antibodies), different responses dependent on the target organ, dose independence, and a striking relationship to the preexisting systemic anti-Ad neutralizing antibody titer.

MATERIALS AND METHODS

Vectors. Three different clinical-grade Ad vectors were used: Ad_{GV}CD.10, Ad_{GV}CFTR.10, and Ad_{GV}VEGF121.10 (all produced by GenVec, Inc., Rockville, Md.). All are $E1^{-}$ $E3^{-}$ replication-deficient vectors based on the Ad serotype 5 (Ad5) genome (18, 19, 21, 41). All carry an expression cassette in the E1 position (right to left) containing the cytomegalovirus early-immediate enhancer-promoter, an artificial splice sequence, the transgene, and the simian virus 40 poly(A)-stop signal (18, 19, 21, 41). Ad_{GV}CD.10 expresses the *Escherichia coli* cytosine deaminase gene (18), Ad_{GV}CFTR.10 expresses the human CF transmembrane conductance regulator (CFTR) cDNA (19), and Ad_{GV}VEGF121.10 expresses the human vascular endothelial growth factor 121 cDNA (21). All Ad vectors were propagated in 293 cells, purified by CsCl density purification, dialyzed, and stored at −70°C (98). All lots fulfilled the in vitro and in vivo safety criteria established by the Food and Drug Administration Bureau of Biologics (FDA BB) for clinical-grade Ad vector preparations (FDA BB-IND 5702, 6442, 6950, and 7381), including the criteria of being free of endotoxin and infectious agents and containing ≤1 replication-competent adenovirus for the total dose to be delivered (18, 19, 21, 41).

Each Ad vector preparation was characterized as to particle units (PU; on the basis of physical particle concentration determined from the absorbance at 260 nm and the extinction coefficient for Ad [9.09×10^{-12} ml × particles^{-1} cm^{-1}] [82]) and PFU (98). The studies of individuals with CF, normal subjects, and those with metastatic colon cancer were based on dosage in PFU; based on a policy decision by the FDA BB for all future Ad gene transfer studies, the coronary artery disease study was based on dosage in PU. Because anti-Ad neutralizing antibodies are directed against the capsid of the Ad vector and not vector activity, the most relevant dose parameter is PU. In this context, all doses have been converted to PU (Table 1 presents PU and PFU characteristics for each Ad vector lot used). Since the analyses of PU and PFU vary ± 20% (data not shown), for convenience in graphing the data, all PU-to-PFU ratios are rounded off to the nearest 10 U (Table 1).

Study design. All clinical studies were approved by the local Institutional Review Boards and Biosafety Committees, the National Institutes of Health Recombinant DNA Advisory Committee, and the Food and Drug Administration. After informed consent was obtained, all studies were initiated with a baseline evaluation to determine eligibility, baseline parameters for safety evaluation, and serum for baseline anti-Ad neutralizing antibodies. This was followed by the vector administration which differed in each protocol (Table 2). All of the complete protocols are on file and available at the Office of Recombinant DNA Activities, National Institutes of Health, Bethesda, Md. The protocols for the q3mo × 3 (every 3 months, three times) repetitive administration study of the Ad_{GV}CFTR.10 vector to individuals with cystic fibrosis and for repetitive administration (q1wk × 2 [once a week, twice]) of the Ad_{GV}CD.10 vector colon cancer metastatic to the liver are also available in the literature (18, 19).

(i) Normal, intradermal. The trial seeks to evaluate the host responses to intradermal administration of the first generation Ad_{GV}CD.10 vector to normal individuals. Each subject was administered the Ad_{GV}CD.10 vector in escalating doses (total dose of 8×10^{7} to 8×10^{9} PU in log increments, $n = 2$ per group) in a volume of 100 μl by the intradermal route with a 26-gauge needle to two different sites, each site receiving 50% of the total dose (Table 2). Six individuals, all males 34 ± 4 years of age participated in the study. The subjects were considered to be healthy on the basis of medical history, physical examination, and standard biochemical, radiographic, and pulmonary function tests.

(ii) CF, intrabronchial. The studies consisted of two parts, A and B. Both evaluated repetitive administration of the Ad_{GV}CFTR.10 vector to the airway epithelium of individuals with CF but differed in the interval between doses. All subjects in parts A and B had CF diagnosed by clinical manifestations, genetic analysis, and/or positive sweat chloride test.

In part A, the Ad_{GV}CFTR.10 vector was administered by endobronchial spray to individuals with CF q3mo × 3. Part A included seven cohorts of 2 individuals each, for a total of 14 individuals (12 males and 2 females, ages 29 ± 2 years; average forced expiratory volume in 1 s [FEV1] of 53% ± 4% predicted). Each cohort was assigned a dose of the Ad_{GV}CFTR.10 vector from 3×10^{7} to 2×10^{10} PU (in half-log increments [Table 2]). The Ad_{GV}CFTR.10 vector was sprayed through a fiberoptic bronchoscope within a lobar bronchus every 3 months, at days 1, 91, and 181. For example, the two individuals in the 3×10^{7}-PU dose group received 10^{7} PU of the Ad_{GV}CFTR.10 vector at three sites, for a total dose of 3×10^{7} PU on day 1, and again at days 91 and 181.

In part B of the protocol, two individuals received the Ad_{GV}CFTR.10 vector in similar fashion as in part A, but q2wk × 4, at one dose ($3 \times 10^{9.5}$ PU [Table 2]). Part B included two individuals (two males, ages 22 and 25 years). Individuals 1 was also included in part A of the protocol (individual 13; receiving a dose of 2×10^{10} PU 4 months prior to the initiation of part B [Table 2]). The two individuals in part B had FEV1 of 44 and 74% predicted, respectively.

(iii) Colon cancer, administration to liver metastasis. The trial is a prodrug study to evaluate the toxicity and biologic efficacy following direct administration of an Ad vector coding for the *E. coli* cytosine deaminase gene (Ad_{GV}CD.10) to hepatic metastases of biopsy proven colorectal carcinoma with concomitant oral administration of the prodrug 5-fluorocytosine. The study is based on the knowledge that the cytosine deaminase gene converts the prodrug 5-fluorocytosine to the potent chemotherapeutic agent 5-fluorouracil. Individuals (five males, 70 ± 3 years old) with biopsy-proven diagnosis of colon cancer and two or more liver metastases scheduled for laparotomy (for resection of liver metastasis, hepatic artery catheter placement, and/or other procedures) as part of their regular treatment for colon cancer were divided into three dose groups, each group to receive the Ad_{GV}CD.10 vector, from a total dose of 8×10^{8} to 8×10^{9} PU administered (50% of the total dose) on day 1, and 50% of the total dose again on day 7 (Table 2). On day 1, the Ad_{GV}CD.10 vector was administered under computer tomography guidance in four equally divided aliquots to the same liver metastasis, each with a separate entry into the metastatic tumor. Each aliquot was contained in a volume of 100 μl. Seven days later, the Ad_{GV}CD.10 vector was administered in an identical fashion in four equally divided aliquots (in 100 μl, under computer tomography guidance) to the same metastasis. Oral 5-fluorocytosine (total of 200 mg/kg of body weight per day in four equally divided doses) was started on day 2 and continued until the day prior to laparotomy. The laparotomy for removal of the metastases was performed on days 11 to 15.

(iv) Coronary artery disease, intramyocardial. The trial is designed to evaluate myocardial angiogenesis induced by direct intramyocardial administration of the Ad_{GV}VEGF121.10 vector to individuals with severe coronary artery disease. The

TABLE 2. Study population, doses, intervals, and sites of vector administration

Group	Vector	Site of administration	Individual no.	Age (yr)	Sex[f]	Total dose for each administration		No. of administrations	Interval between administrations
						PFU	PU		
Normal	Ad_{GV}CD.10	Skin	1	23	M	2×10^6	8×10^7	1	—[c]
			2	25	M	2×10^6	8×10^7	1	—
			3	42	M	2×10^7	8×10^8	1	—
			4	40	M	2×10^7	8×10^8	1	—
			5	46	M	2×10^8	8×10^9	1	—
			6	29	M	2×10^8	8×10^9	1	—
CF									
Part A	Ad_{GV}CFTR.10[a]	Airway epithelium	1	37	M	3×10^6	3×10^7	3	3 mo
			2	24	M	3×10^6	3×10^7	3	3 mo
			3	22	M	$3 \times 10^{6.5}$	$3 \times 10^{7.5}$	1	—
			4	26	M	$3 \times 10^{6.5}$	$3 \times 10^{7.5}$	3	3 mo
			5	33	M	3×10^7	3×10^8	1	—
			6	24	F	3×10^7	3×10^8	3	3 mo
			7	24	M	$3 \times 10^{7.5}$	$3 \times 10^{8.5}$	3	3 mo
			8	48	F	$3 \times 10^{7.5}$	$3 \times 10^{8.5}$	1	—
			9	25	M	3×10^8	3×10^9	3	3 mo
			10	20	M	3×10^8	3×10^9	2	3 mo
			11	38	M	$3 \times 10^{8.5}$	$3 \times 10^{9.5}$	3	3 mo
			12	42	M	$3 \times 10^{8.5}$	$3 \times 10^{9.5}$	3	3 mo
			13[e]	25	M	2×10^9	2×10^{10}	3	3 mo
			14	17	M	2×10^9	2×10^{10}	3	3 mo
Part B	Ad_{GV}CFTR.10	Airway epithelium	1[b]	25	M	$3 \times 10^{8.5}$	$3 \times 10^{9.5}$	4	15 days
			2	22	M	$3 \times 10^{8.5}$	$3 \times 10^{9.5}$	4	15 days
Metastatic colon cancer	Ad_{GV}CD.10	Metastatic liver tumors	1	63	M	2×10^7	8×10^8	2	1 wk
			2	65	M	2×10^7	8×10^8	2	1 wk
			3	75	M	2×10^7	8×10^8	2	1 wk
			4	75	M	2×10^8	8×10^9	2	1 wk
			5	72	M	2×10^8	8×10^9	2	1 wk
Coronary artery disease	Ad_{GV}VEGF121.10	Intramyocardial	1	60	M	10^7	4×10^8	1	—
			2	56	M	10^7	4×10^8	1	—
			3	64	M	10^7	4×10^8	1	—
			4	66	M	$10^{7.5}$	$4 \times 10^{8.5}$	1	—
			5	60	M	$10^{7.5}$	$4 \times 10^{8.5}$	1	—
			6	66	M	$10^{7.5}$	$4 \times 10^{8.5}$	1	—
			7	65	F	10^8	4×10^9	1	—
			8	52	F	10^8	4×10^9	1	—
			9	46	M	10^8	4×10^9	1	—
			10	45	M	$10^{8.5}$	$4 \times 10^{9.5}$	1	—
			11	51	F	$10^{8.5}$	$4 \times 10^{9.5}$	1	—

[a] Individuals 1 to 8 received lot 0056-0002; individuals 9 to 14 received lot 0045-0007; (Table 1).
[b] Individual 13 in part A was the same as individual 1 in part A; the vector in part B was first administered 4 months after the last vector administration in part A.
[c] —, administered one time only.
[d] Administered two times only.
[e] The first dose given to individual 13 was 3×10^{10} PU; subsequent doses were 2×10^{10} PU.
[f] M, male; F, female.

trial is a dose-escalating (4×10^8 to $4 \times 10^{9.5}$ PU) safety study of 11 individuals (eight males and three females; 57 ± 2 years old) divided into four dose groups. All had severe coronary artery disease diagnosed by medical history, electrocardiogram, treadmill stress test, echocardiogram, ^{99m}Tc-sestamibi flow studies, and cardiac catheterization with angiography. The Ad_{GV}VEGF121.10 vector was administered directly to the myocardium following bypass surgery using a 28-gauge needle at ≤5-mm vertical depth in 10 divided doses of 100 μl at 1.0- to 1.5-cm intervals in a myocardial region in a coronary artery distribution that was not bypassed.

Anti-Ad5 neutralization antibody titers. Anti-Ad5 neutralizing antibody titers were measured by the ability of serum to prevent infection of A549 cells (CCL 185; American Type Cell Culture Collection, Rockville, Md.) by wild-type Ad5 as previously described (72, 76). Briefly, the A549 cells were cultured in improved Eagle's minimum essential medium containing 10% fetal bovine serum, 2 mM glutamine, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Cells were seeded in 96-well plates (Falcon 3072; Becton Dickinson, Lincoln Park, N.J.) at a density of 3×10^4 cells/well in a volume of 200 μl of medium. Serum samples were heat inactivated at 55°C for 40 min, diluted serially, and added to a 96-well plate (Falcon 3077; Becton Dickinson). An amount of Ad5 equivalent to a multiplicity of infection of 1 was added to the serum, and the plates were incubated for 1 h at 37°C. The mixture was subsequently added to the A549 cells, and the cells were incubated until the serum-free control wells exhibited >95% cytopathic effect (typically 6 to 8 days) evaluated by methylene blue staining (EM Diagnostic Systems, Gibbstown, N.J.). The neutralizing antibody titer (per 5 μl of serum) was calculated as the product of the reciprocal of the initial dilution times the reciprocal of the dilution in the last well showing <95% cytopathic effect. All assays were performed in triplicate, and data are presented as the mean of three determinations. Human serum known to have anti-Ad5 neutralizing antibodies was used as a positive control, and human serum known to lack anti-Ad neutralizing antibodies was used as a negative control.

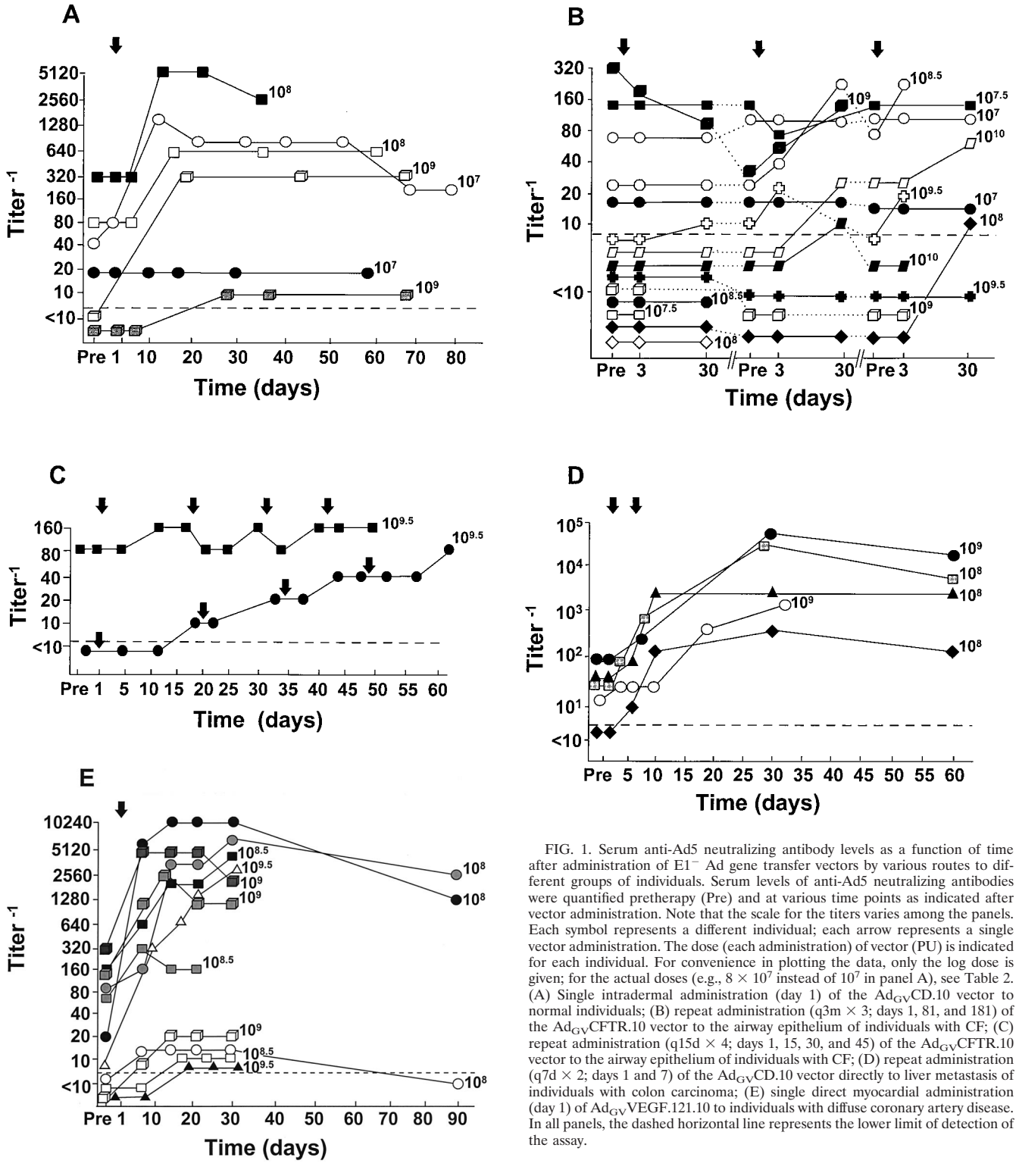


FIG. 1. Serum anti-Ad5 neutralizing antibody levels as a function of time after administration of E1$^-$ Ad gene transfer vectors by various routes to different groups of individuals. Serum levels of anti-Ad5 neutralizing antibodies were quantified pretherapy (Pre) and at various time points as indicated after vector administration. Note that the scale for the titers varies among the panels. Each symbol represents a different individual; each arrow represents a single vector administration. The dose (each administration) of vector (PU) is indicated for each individual. For convenience in plotting the data, only the log dose is given; for the actual doses (e.g., 8×10^7 instead of 10^7 in panel A), see Table 2. (A) Single intradermal administration (day 1) of the Ad_{GV}CD.10 vector to normal individuals; (B) repeat administration (q3m $\times$ 3; days 1, 81, and 181) of the Ad_{GV}CFTR.10 vector to the airway epithelium of individuals with CF; (C) repeat administration (q15d $\times$ 4; days 1, 15, 30, and 45) of the Ad_{GV}CFTR.10 vector to the airway epithelium of individuals with CF; (D) repeat administration (q7d $\times$ 2; days 1 and 7) of the Ad_{GV}CD.10 vector directly to liver metastasis of individuals with colon carcinoma; (E) single direct myocardial administration (day 1) of Ad_{GV}VEGF.121.10 to individuals with diffuse coronary artery disease. In all panels, the dashed horizontal line represents the lower limit of detection of the assay.

Statistical analysis. Error estimates are presented as mean ± standard deviation. Comparison of the frequency of undetectable baseline anti-Ad5 neutralizing antibodies among the four groups was made by chi-square test. For comparison of the mean ages among the groups of individuals studied, the data were evaluated by an analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) post-hoc test for unequal sample sizes. To test the difference between age and FEV1 by detectable baseline anti-Ad5 within the CF group, a Welch modified Student two-sample *t* test for unequal variances was used. Due to the nonnormal distribution of the different parameters evaluated (route, age, dose, and disease state), nonparametric statistical analyses were done to evaluate the difference in anti-Ad neutralizing antibody response for the different routes of vector administration and the anti-Ad neutralizing antibody response in relation to baseline anti-Ad titer and dose of vector administered. For the difference in distribution of systemic anti-Ad5 neutralizing antibody response for the four routes of vector administration, the data were ranked and analyzed by Kruskal-Wallis ANOVA. The relationship between baseline systemic anti-Ad5

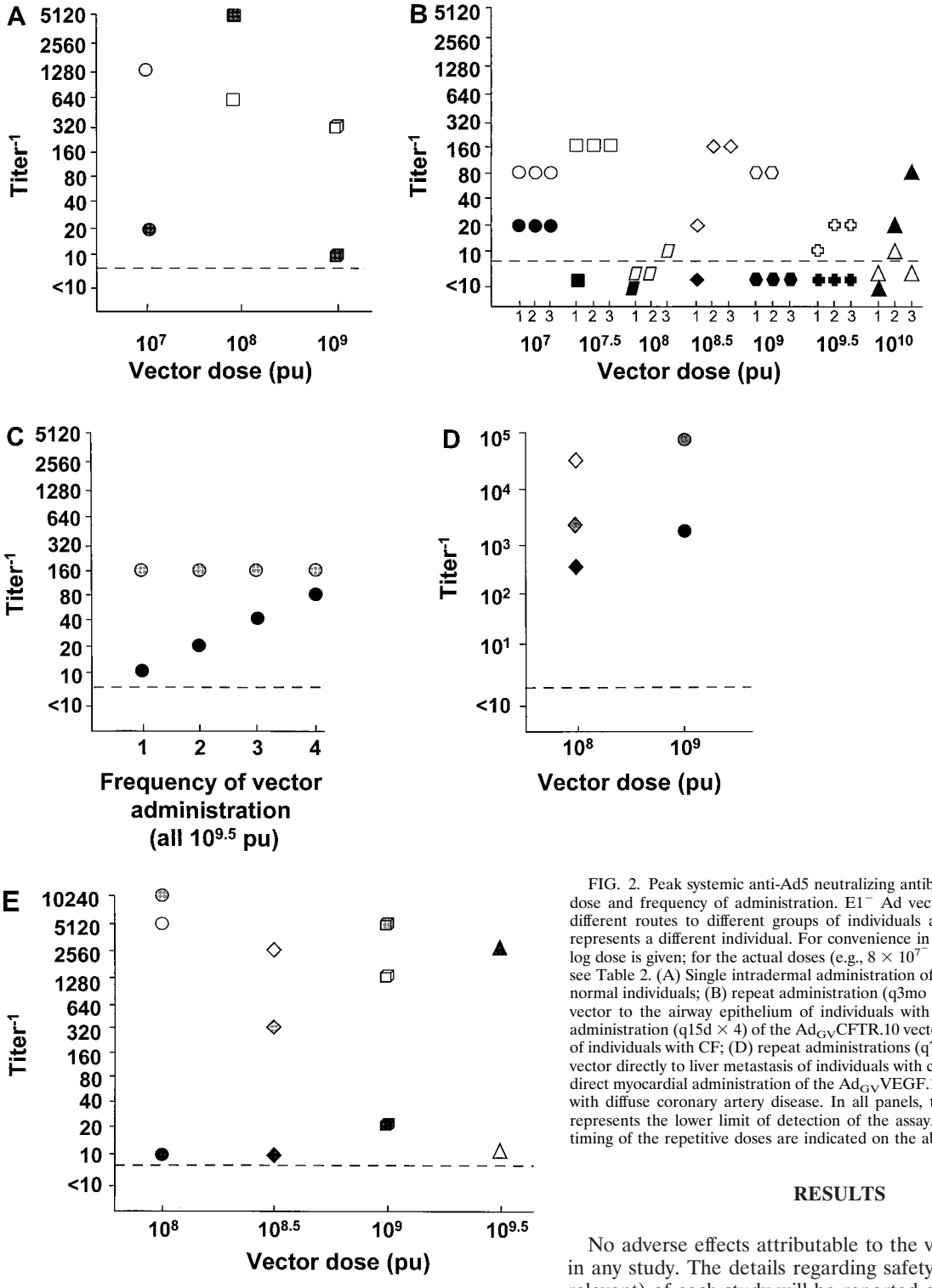


FIG. 2. Peak systemic anti-Ad5 neutralizing antibody levels as a function of dose and frequency of administration. E1$^-$ Ad vectors were administered by different routes to different groups of individuals as indicated. Each symbol represents a different individual. For convenience in plotting the data, only the log dose is given; for the actual doses (e.g., $8 \times 10^{7-}$ instead of 10^7 in panel A), see Table 2. (A) Single intradermal administration of the Ad_{GV}CD.10 vector to normal individuals; (B) repeat administration (q3mo $\times$ 3) of the Ad_{GV}CFTR.10 vector to the airway epithelium of individuals with cystic fibrosis; (C) repeat administration (q15d $\times$ 4) of the Ad_{GV}CFTR.10 vector to the airway epithelium of individuals with CF; (D) repeat administrations (q7d $\times$ 2) of the Ad_{GV}CD.10 vector directly to liver metastasis of individuals with colon carcinoma; (E) single direct myocardial administration of the Ad_{GV}VEGF.121.10 vector to individuals with diffuse coronary artery disease. In all panels, the dashed horizontal line represents the lower limit of detection of the assay. For panels B and C, the timing of the repetitive doses are indicated on the abscissa.

neutralizing antibodies and dose of vector administered with the peak systemic anti-Ad5 neutralizing antibody response was measured by the Spearman rank correlation coefficient. Statistical significance was taken for P values of less than 0.05. The statistics analysis was carried out by using the programs S-plus 4.5 for Windows (Mathsoft, Inc., Seattle, Wash.) and SAS (SAS Institute, Cary, N.C.). For individuals without detectable neutralizing antibodies at baseline, fold increase over baseline was determined by using the titer of 9 since 10 is the lowest detectable titer with the assay.

RESULTS

No adverse effects attributable to the vector were observed in any study. The details regarding safety and efficacy (where relevant) of each study will be reported elsewhere.

Preexisting anti-Ad neutralizing antibodies. Evaluation of the different groups of individuals (normal, CF, metastatic colon cancer, and coronary artery disease) demonstrated a broad range of preexisting anti-Ad neutralizing antibody titers, although the differences among the various groups were not significant (Fig. 1). No detectable serum anti-Ad neutralizing antibodies (titer of <10) were detected in 33% (2 of 6) of normal subjects, 67% (10 of 15) of individuals with CF (all

individuals in group A plus one individual in group B; individual 1 in group B previously participated in group A, [Table 2]), 20% (1 of 5) of individuals with metastatic colon cancer, and 27% (3 of 11) of individuals with coronary artery disease. Although a larger proportion of individuals with CF had no detectable pretherapy neutralizing antibodies (>2 times that of any other group), this level did not achieve statistical significance ($P = 0.21$ [chi-square test] compared to all other groups).

While the age of the CF group was lower than those of the cancer and coronary artery groups ($P < 0.001$ [multiple comparison, ANOVA, and Tukey HSD post-hoc test for unequal sample sizes], both comparisons), it was no different from that of the normal group ($P = 0.62$). Within the CF group, the average age of those with undetectable anti-Ad neutralizing antibodies was no different from that of those with detectable antibodies [30 ± 10 years versus 26 ± 6 years; $P = 0.38$ [Student's two-sample t test for unequal variances]), and there was no difference among those in the CF group with nondetectable and detectable antibodies in the average FEV1 (54% ± 17% predicted versus 52% ± 11% predicted, $P = 0.79$ [Student's two-sample t test for unequal variances]).

Systemic anti-Ad5 neutralizing antibody responses to vector administration. Evaluation of systemic anti-Ad5 neutralizing antibodies revealed a variable response depending on the different routes of administration and/or the disorders of the recipients (Fig. 1). The least responses were in individuals with CF after intrabronchial administration of the Ad_{GV}CFTR.10 vector (Fig. 1B and C), and the most robust systemic anti-Ad5 neutralizing antibody responses were observed after administration of Ad_{GV}CD.10 to liver metastasis of individuals with colon cancer (Fig. 1D). Administration of the Ad vectors by the intradermal and intramyocardial routes resulted in a variable response, with minimal to no neutralizing antibody response in 2 of 6 individuals in the normal trial and 4 of 11 in the coronary artery disease trial (Fig. 1A and E). The data suggest that there is a difference in the distribution of peak anti-Ad5 neutralizing antibody titer according to the route of administration ($P = 0.0023$ [Kruskal-Wallis test]). In general, for those individuals in whom antibody titers were increased significantly above baseline (in all trials), the peak anti-Ad neutralizing antibody levels were detected by 2 to 4 weeks after vector administration, with levels significantly higher than pre-vector administration levels still present 8 to 12 weeks after Ad vector administration.

(i) Normal (Ad_{GV}CD.10, intradermal). For the six individuals studied, one (8×10^7 PU) had no response, and one (8×10^9 PU) had only a minimal increase in serum systemic anti-Ad5 neutralizing antibody titer from the baseline (Fig. 1A). In contrast, the other four individuals responded to vector administration with significantly increased levels of anti-Ad5 neutralizing antibodies above baseline, with peak levels observed by the second week after the Ad_{GV}CD.10 vector was administered. The largest increase was observed in two individuals (8×10^8 and 8×10^9 PU) with 32- and 35-fold increases in the neutralizing titer, respectively.

(ii) CF (Ad_{GV}CFTR.10, intrabronchial). Strikingly, no or minimal systemic anti-Ad5 neutralizing antibodies were observed in individuals with CF, even after repeated lung administration of the Ad_{GV}CFTR.10 vector. In part A of the protocol, no significant increase in antibody titers were observed after the first vector administration at any dose (Fig. 1B). Likewise, after the second vector administration, only minimal increases of anti-Ad5 neutralizing antibody titers were observed. The maximum increase (eightfold) was observed in one subject at a dose of $3 \times 10^{8.5}$ PU by the third week after the second vector administration. Following the third vector administration, only minimal (twofold maximum) increases in anti-Ad5 antibody titer were seen in four individuals. In part B of the CF protocol (Fig. 1C), one individual responded with a minimal increase in the anti-Ad5 titer, with successive twofold increase in antibody titers after each lung administration of the Ad_{GV}CFTR.10 vector. The individual 2 had no change in anti-Ad antibody titer throughout the study, despite four administrations of the vector 2 weeks apart. Importantly, this same individual had also participated in part A of the CF trial. In part A, he received three lung administrations of Ad_{GV}CFTR.10 (2×10^{10} PU q3mo × 3), followed 4 months later, in part B, by four lung administrations ($3 \times 10^{9.5}$ PU q15d × 4). Interestingly, even after a total of seven intrabronchial administrations of the AdCFTR.10 vector, the systemic neutralizing antibody titer increased only minimally, with the increase observed only in part A of the trial (Fig. 1B and C).

(iii) Colon carcinoma metastatic to liver (Ad_{GV}CD.10). In striking contrast to intrabronchial administration of an Ad vector, most individuals receiving intratumor (colon carcinoma metastatic to liver) administration of an Ad vector responded with a vigorous systemic anti-Ad5 neutralizing antibody response, with peak antibody levels seen in all individual by the fourth week after vector administration (Fig. 1D). The maximum titers observed (at doses of 8×10^8 and 8×10^9 PU) reached $>10^4$. The lowest antibody increase was noted in an individual at a dose of 8×10^8 PU, whose peak level reached 640. In the other two individuals in the study (receiving doses of 8×10^8 and 8×10^9 PU), anti-Ad5 antibody levels increased from $<10^2$ at baseline to 10^3 by the fourth week following vector administration.

(iv) Coronary artery disease (Ad_{GV}VEGF.121.10, intramyocardial). Of the 11 individuals studied, 4 (receiving 4×10^8, $4 \times 10^{8.5}$, 4×10^9, and $4 \times 10^{9.5}$ PU, respectively) had no significant increase in anti-Ad5 neutralizing antibody titer from baseline, and a fifth individual ($4 \times 10^{8.5}$ PU) had only a maximum fourfold increase in antibody titer over baseline. The other six individuals had a more vigorous response, with anti-Ad5 antibody titers peaking by the second week after vector administration at 10^3 to 10^4. The highest titer was in an individual receiving 4×10^8 PU, with a 512-fold increase in the titer from baseline (Fig. 1E).

Relationship of antibody responses to dose and preexisting antibody titers. Remarkably, analysis of the peak anti-Ad5 neutralizing antibody responses to administration of the Ad vectors demonstrated no relationship with the dose of vector administered (Fig. 2). Further, the only apparent dependency on frequency of administration was in one individual with CF receiving four administrations of the Ad_{GV}CFTR.10 vector ($3 \times 10^{8.5}$ PU) (Fig. 2C). Among all other individuals, whether peak antibody responses among different individuals receiving different doses or peak antibody responses among the different individuals receiving repetitive doses were compared, no dose dependency was observed (skin route, correlation coefficient −0.35, $P = 0.48$; lung route, correlation coefficient −0.39, $P = 0.14$; intraliver tumor route, correlation coefficient −0.09, $P = 0.82$; intramyocardial route, correlation coefficient −0.22, $P = 0.52$ [all Spearman's rank correlation]). This lack of dose dependency was also true when the analysis was carried out by examining the increase in peak titers (over baseline titers) compared to dose (skin route, correlation coefficient 0.23, $P = 0.64$; lung route, correlation coefficient 0.23, $P = 0.39$; intraliver tumor route, correlation coefficient −0.39, $P = 0.43$; intramyocardial route, correlation coefficient −0.11, $P = 0.73$ [all Spearman's rank correlation]). Finally, the lack of dose dependency also held when the individuals with no preexisting

anti-Ad neutralizing antibody titers were eliminated from the analysis (not shown; correlation coefficient 0.3, $P = 0.13$).

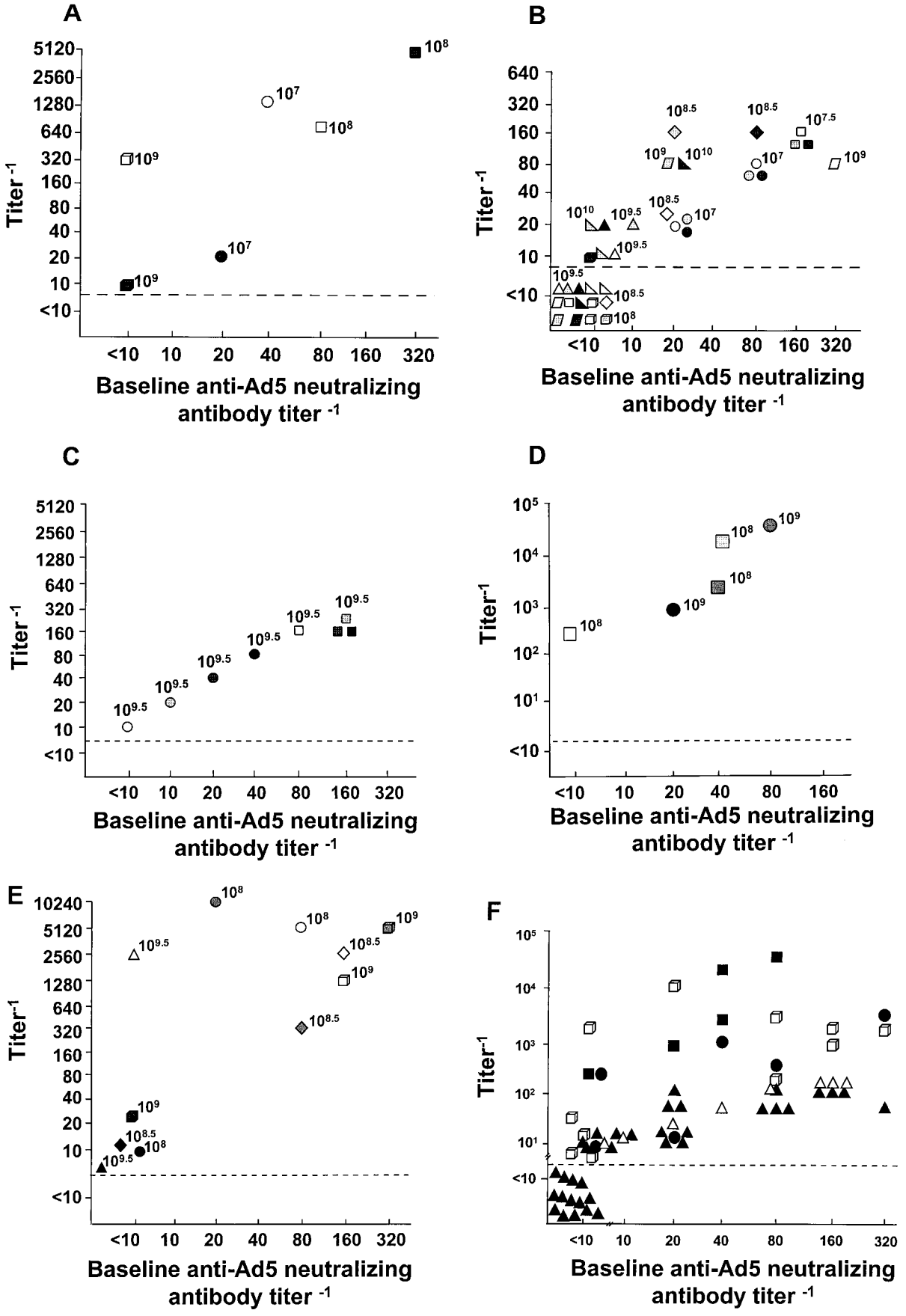
In contrast to the lack of correlation of peak neutralizing antibody titers with dose, there was a remarkable correlation of peak neutralizing antibody titers with the baseline anti-Ad5 neutralizing antibody titers (Fig. 3). This was true for intradermal administration to normal subjects (correlation coefficient 0.84, $P = 0.036$ [Spearman's rank correlation] [Fig. 3A]), repetitive (q3mo $\times$ 3) airway administration to individuals with CF (correlation coefficient for first administration 0.90, $P = 0.0001$; correlation coefficient for second administration 0.90, $P = 0.0002$; correlation coefficient for third administration 0.90, $P = 0.0001$ [Fig. 3B]), repetitive (q2wk $\times$ 4) airway administration to individuals with CF (correlation coefficient 0.91, $P = 0.0001$ [Fig. 3C]), repetitive administration (q3mo $\times$ 3, followed 4 months later by q15d $\times$ 4) to the airway epithelium of one individual with CF who participated in parts A and B of the CF trial (correlation coefficient 0.94, $P = 0.02$ [Fig. 3D]), intratumor administration to individuals with colon cancer metastatic to liver (correlation coefficient 0.98, $P = 0.0003$ [Fig. 3D]), and intramyocardial administration to individuals with coronary artery disease (correlation coefficient 0.73, $P = 0.011$ [Fig. 3E] [all analyses by Spearman's rank correlation]). Finally, analysis of all of the data together demonstrated that despite the disparity in peak titers, there was still a significant correlation of peak neutralizing antibody titers with the baseline titers (correlation coefficient 0.77, $P < 0.0001$ [Fig. 3F]). This was also true when we analyzed by examining the increase in peak titers (over baseline levels) compared to baseline titers (correlation coefficient 0.34, $P = 0.034$).

DISCUSSION

Neutralizing anti-Ad antibodies, representing a subset of total serum anti-Ad antibodies, function by preventing the virus from binding to cell membrane receptors and/or preventing translocation of the internalized Ad from the endosome into the cytoplasm (13, 128, 129). Based on the experience of administration of Ad gene transfer vectors to experimental animals, the general principle has evolved that the administration of Ad vectors to the immune naive host almost invariably evokes neutralizing antibodies directed against the Ad (11, 25, 27, 31, 35, 44, 47–49, 51–53, 57, 58, 62, 63, 65, 66, 72, 76, 77, 80, 101, 103, 104, 108–110, 114, 118–121, 124, 127, 131, 132, 134–138). However, the present study demonstrates that at the doses used (3×10^7 to 2×10^{10} PU), with the caveat that there is a significant difference in body mass of humans and the experimental animals typically used in gene transfer studies, humans differ from experimental animals in responding to Ad vectors. While humans are capable of increasing systemic anti-Ad neutralizing antibody titers following administration of Ad vectors, the systemic humoral response to Ad vectors in humans is variable, from no response to a vigorous response with neutralizing titers of $>10^4$. Whereas naive experimental animals respond with systemic anti-Ad neutralizing antibodies following administration of Ad vectors to all organs and routes evaluated (except for the retina and brain [8, 90]), the present study demonstrates that (i) intrabronchial administration of an Ad vector to individuals with CF results in no or minimal systemic anti-Ad neutralizing antibodies, despite repetitive administration three times over a 6-month period or four times over a 2-month period; (ii) in contrast, at the same or lower doses, administration of Ad vectors to liver tumors (intratumoral) of individuals with metastatic colon cancer, the skin (intradermal) of normal individuals, or the heart (intramyocardial) of individual with coronary artery disease evokes a variable anti-Ad humoral response, from little or no response to striking anti-Ad neutralizing antibody titers of $>10^4$; (iii) very differently than for experimental animals (108, 110, 137), the humoral response to Ad vectors in humans at the doses administered in these studies is dose independent; and (iv) whereas immune naive (i.e., no preexisting systemic anti-Ad neutralizing antibodies) experimental animals develop a humoral anti-Ad response within 2 to 4 weeks after Ad vector administration independent of route (11, 25, 27, 31, 35, 44, 47–49, 51–53, 57, 58, 62, 63, 65, 66, 72, 76, 77, 80, 101, 103, 104, 108–110, 114, 118–121, 124, 127, 131, 132, 134–138), the anti-Ad vector response in humans is strikingly dependent on the preexisting anti-Ad neutralizing antibody titer. Together, these observations demonstrate some of the pitfalls of relying on experimental animal studies to predict human host responses to Ad gene transfer vectors and lead to the important conclusion that the design of human Ad gene transfer studies should take into account the preexisting anti-Ad humoral immune status of the potential participants in assessing the safety and efficacy of Ad vectors.

Humoral response to Ad vectors in experimental animals. Anti-Ad neutralizing antibody responses to administration of Ad vectors to naive animals is dose dependent (108, 110, 137) and is dependent on the route of administration, with the highest Ad neutralization observed after intravenous administration (11, 25, 27, 31, 35, 44, 47–49, 51–53, 57, 58, 62, 63, 65, 66, 72, 76, 77, 80, 101, 103, 104, 108–110, 114, 118–121, 124, 127, 131, 132, 134–138). By most routes, administration of Ad vectors to naive animals is followed by a systemic neutralizing antibody response which peaks within 2 to 3 weeks and decreases by the second or third month after vector administration. No systemic anti-Ad antibody responses are observed following administration to the retina, and a minimal neutralizing antibody response has been reported after administration to the brain (7, 90).

Humoral response to wild-type Ad in humans. The data regarding humoral responses to wild-type Ad must be viewed with caution in regard to the present study in that Ad gene transfer vectors are $E1^-$, whereas the expression of the E1 genes following wild-type Ad infection likely influences the spectrum and quantity of expression of Ad gene products presented to the immune system for humoral responses. Importantly, all vector preparations in the present study had ≤ 1 replication-competent Ad per dose administered, and thus the expression of Ad5 E1 genes does not play a role in the anti-Ad neutralizing antibody host responses observed (18, 19, 21, 41). Given this caveat, there are data for normal subjects and individuals with CF regarding anti-Ad systemic neutralizing antibodies following infection with wild-type Ad. Most normal adults (>85%) have systemic anti-Ad antibodies (total antibodies, not necessarily neutralizing) against several of the most frequently encountered Ad serotypes, including subgroup C, serotype 5, the base Ad used for the vectors in the present study (43, 50, 102). Early infection with wild-type Ad in normal humans is followed by a systemic serotype-specific immunoglobulin G (IgG) and IgM anti-Ad antibody response (81). Twenty-seven to 57% of normals are reported to have anti-Ad5 serum neutralizing antibodies (91, 104). Anti-Ad neutralizing antibodies usually develop within 2 weeks of primary infection with wild-type Ad, and if reinfection does not occur, neutralizing antibodies generally decline to undetectable levels over a 2-year period (17, 46). Enteric administration of Ad serotypes 1, 2, 4, 5, 7, and 21 to normal humans also triggers the formation of neutralizing antibodies (26, 84, 105, 106). Based on these studies, an oral vaccine composed of wild-type Ad4 and Ad7 is routinely administered to healthy, military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recruits followed by protective systemic neutralizing antibodies (115).

As in normal individuals, the prevalence of systemic anti-Ad5 (total, not specifically neutralizing) antibody titers in individuals with CF are reported to be high, 97% having anti-Ad5 IgG antibodies and 53% having systemic anti-Ad5 IgM antibodies (96). Children with CF have variability in the prevalence of systemic neutralizing antibodies against various Ad serotypes, with neutralizing antibodies against Ad5 in 17% (91). The prevalence of systemic anti-Ad5 neutralizing antibodies in the healthy mothers of the same children was 49%.

Humoral responses to administration of Ad vectors to the respiratory epithelium in humans. Systemic anti-Ad neutralizing antibody responses to $E1^-$ Ad vectors has been evaluated in a variety of clinical trials in which the vector has been administered to the respiratory epithelium. Administration of an Ad5-based vector carrying the normal CFTR cDNA to the nasal epithelium followed by administration to the airway epithelium of four individuals resulted in no change in systemic neutralizing antibody titers (20). Single nasal administration of an Ad5-based vector containing the CFTR cDNA to 12 individuals with CF was followed by no change in anti-Ad neutralizing antibody titer in 11, with only one individual (at the highest dose, 10^{10} PFU) having a 16-fold increase in the titer from baseline (60). Repeat (four to five times) nasal administration of an Ad2-based vector containing the normal CFTR cDNA to six individuals with CF resulted in minimal systemic neutralizing antibody response, with maximal increases in titer of four- to eightfold above baseline (139). Nasal instillation followed by lung (aerosol) administration of an Ad5-based vector carrying the normal CFTR cDNA to six individuals with CF resulted in no increase in anti-Ad neutralizing antibodies (7). These observations of minimal humoral responses to administration of Ad vectors to the respiratory epithelium in CF is likely not because of a defect in immunity in CF, in that individuals with CF have no evidence of increased susceptibility to respiratory viral infections (91, 93, 125). Consistent with these observations in CF, direct injection of an Ad5-based vector expressing the *E. coli* β-galactosidase gene to endobronchial tumors in four individuals with lung cancer was followed by a minimal systemic neutralizing antibody response, at most a fourfold increase over baseline (32). Together with the data in the present study, these observations strikingly demonstrate that the anti-Ad neutralizing antibody response elicited after administration of Ad vectors to the human respiratory epithelial surface is minimal compared with other routes of administration, a conclusion that is very different than the experience in experimental animals where the anti-Ad humoral response is invariably robust following Ad vector administration to the respiratory epithelium (53, 72, 76, 120, 121, 134, 137).

Humoral responses to administration of Ad vectors in sites other than the respiratory epithelium in humans. Other than the present study, there are very few data available relating the administration of $E1^-$ Ad vectors to sites in humans other than the respiratory epithelium. Intrapleural administration of an Ad5-based vector expressing the *Herpes simplex* virus thymidine kinase gene to 21 individuals with localized mesothelioma was followed by a systemic but variable neutralizing antibody response in most individuals (83), and administration of a similar vector to malignant gliomas resulted in the development of systemic neutralizing antibodies (3). Since the Ad capsid is the major factor in inducing anti-Ad antibodies against Ad vectors, it is instructive to review the responses of normal humans to protein components of Ad. Intramuscular and intradermal administration to normal individuals of soluble antigen from Ad1 or Ad2 (both subgroup C) was followed by the development of anti-Ad1 or anti-Ad2 neutralizing antibodies in 90%, with low levels of neutralizing antibodies detected in most individuals after 1 year (6, 54). Intramuscular administration to normal individuals of Ad5 hexon elicited anti-Ad neutralizing antibodies in 80%, and intramuscular administration of Ad5 fiber elicited anti-Ad neutralizing antibodies in 100% (16). All of those developing neutralizing antibodies were protected when challenged with nasal administration of wild-type Ad5, whereas nonimmunized controls developed symptoms of Ad infection (16).

Factors modulating systemic humoral responses to Ad vectors. An important finding in the present study was the variability of the anti-Ad neutralizing antibody response in the different trials. Differences in the vector per se does not explain the variability of the antibody response, since the three Ad vectors used in the four trials in the present study were all derived from the same Ad5 backbone, and all have identical Ad capsids. Consistent with these observations, when normal individuals with undetectable baseline serum anti-Ad neutralizing antibodies who subsequently had natural wild-type Ad infections (diagnosed by fecal and/or respiratory Ad isolation), a variable proportion developed systemic anti-Ad neutralizing antibodies (29).

The present study was not designed to identify specific Ad capsid epitopes that are recognized by the anti-Ad neutralizing antibodies evoked by the Ad vectors. However, the experience from humans following wild-type Ad infection suggests that the dominant neutralizing antibodies observed against the Ad vectors are against hexon and fiber (16). It has been suggested that Ad vectors also induce neutralizing antibodies against the penton base protein (30).

The data in the present study suggest that the route of administration plays a major role in the systemic anti-Ad neutralizing antibody response, although there are insufficient data to determine whether the underlying disease state modifies the systemic humoral host response to Ad vectors. Administration of the Ad vector to the bronchial epithelium of individuals with CF yielded the lowest antibody response, while direct injection of the Ad vector to colon carcinoma metastases in the liver resulted in the most vigorous antibody response. One factor that may be relevant in regard to minimal responses to bronchial administration of the Ad_{GV}CFTR.10 vector in CF is that the airway epithelium of these individuals is covered by high quantities of mucopurulent secretions which could preclude

FIG. 3. Peak systemic anti-Ad5 neutralizing antibody levels as a function of anti-Ad5 neutralizing antibody titer before vector administration. $E1^-$ Ad vectors were administered by different routes to different groups of individuals. Each symbol represents a different individual. (A) Single intradermal administration of the Ad_{GV}CD.10 vector to normal individuals; (B) repeat administration (q3mo × 3) of the Ad_{GV}CFTR.10 vector to the airway epithelium of individuals with CF. Open symbols, first administration; light gray symbols, second administration; dark gray symbols, third administration. (C) Repeat administration (q15d × 4) of the Ad_{GV}CFTR.10 vector to the airway epithelium of individuals with CF. Open symbols, first administration; light gray symbols, second administration; dark gray symbols, third administration; black symbols, fourth administration. (D) Repeat administration (q7d × 2) of the Ad_{GV}CD.10 vector directly to liver metastasis of individuals with colon carcinoma. (E) Single direct myocardial administration of the Ad_{GV}VEGF.121.10 vector to individuals with diffuse coronary artery disease. (F) Data combined from panels A to E. ●, intradermal, normal, from panel A; ▲, airway, CF, from panel B; △, airway, CF, from panel C; ■, liver, colon carcinoma, from panel D; ❒, heart, coronary artery disease, from panel E. In all panels, the dashed horizontal line represents the lower limit of detection of the assay. For convenience in plotting the data, only the log dose is given; for the actual doses (e.g., 8×10^7 instead of 10^7 in panel A), see Table 2.

efficient Ad-vector infection of the airway epithelial cells (112, 122).

The strong anti-Ad neutralizing antibody response observed in the metastatic colon cancer trial might be explained by the close proximity of the site of vector administration in the intrahepatic tumors to the liver reticuloendothelial system and/or to the vector gaining access to the systemic circulation. We do not have any direct evidence that the vector reached the circulation following intratumoral administration, but this is a possibility, since administration of the $Ad_{GV}CD.10$ vector was performed with a needle which traveled through normal liver tissue, and the metastatic colon cancer tumors are relatively firm, making it difficult to disperse the vector through the tumor. Another factor that may play a role is the damage to tumor cells induced by the destruction of the tumor by the conversion of 5-fluorocytosine to 5-fluorouracil by the cytosine deaminase expressed by the $Ad_{GV}CD.10$ vector.

While some individuals in the intradermal and intramyocardial trials did not raise the anti-Ad neutralizing antibody titer following administration of the Ad vector, most had a significant systemic anti-Ad neutralizing antibody response, with some individuals in the intramyocardial trial having as vigorous response as some individuals in the colon cancer study. Similar to the intrahepatic tumor administration, it is possible that intradermal or intramyocardial administration introduces small amounts of the vector into the systemic circulation and/or exposes the Ad vector to antigen-presenting cells in the local draining lymph nodes. However, other factors must play a role, since not all individuals in these two trials responded with an increase in the antibody titer.

The most striking finding in the present study was the correlation of the peak systemic anti-Ad5 neutralizing antibody response with the level of baseline anti-Ad5 neutralizing titers. Irrespective of route of administration and underlying disorder, we observed that individuals with higher baseline anti-Ad neutralizing antibody titer mounted a higher neutralizing antibody response after vector administration, consistent with a secondary immune response in those individuals previously exposed to Ad of a similar serotype (29, 92, 94).

Relevance to future gene therapy trials. There are several important conclusions from these results that have implications for future gene therapy trials. First, unlike in animal studies (53, 72, 76, 120, 134, 138), repeated respiratory epithelial administration of $E1^-$ Ad vectors is not followed by a strong systemic anti-Ad neutralizing antibody response, i.e., the generation of anti-Ad systemic neutralizing antibodies does not seem to be a major host immune response after repeated respiratory epithelial administration of Ad vectors. This observation suggests that repeat administration of Ad vectors to the lung may be feasible, at least in regard to the hurdle of systemic humoral immunity against Ad vectors, although local mucosal immunity and other factors (e.g., vector genome and epithelial cell Ad receptors) play a more important role in the efficiency of Ad-mediated gene transfer after repeated lung administration (72, 76, 79, 86–88, 111, 134). A caveat to the minimal anti-Ad neutralizing antibody response observed in the CF trial is that the vector was administered to only a limited region of the airways; i.e., doses higher than the one used in this trial ($>2 \times 10^{10}$ PU) might elicit a different systemic anti-Ad neutralizing antibody response in humans. Second, the extent of the observed humoral immune responses to Ad5-based vectors is related to preexisting systemic anti-Ad5 neutralizing antibodies, likely due to prior exposure to wild-type subgroup C Ad. This observation is important for the design of gene therapy strategies, since (i) high anti-Ad neutralizing antibodies may prevent, or significantly reduce, the efficiency of gene transfer upon administration of a similar serotype-based Ad vector (57, 72, 76, 108, 134) and (ii) in the absence of adenovirus replication, the host immune system still recognizes the Ad capsid proteins and mounts an anti-Ad neutralizing antibody response; i.e., for the design of new "stealth" Ad vectors, simply deleting Ad genes (12, 14, 28, 36, 61, 68, 69) will likely not circumvent the anti-Ad humoral response for in vivo gene therapy applications requiring repeated Ad vector administration.

There are several theoretical strategies that might be used in human gene therapy applications to circumvent the host humoral immune responses to Ad vectors. First, sequential administration of different-serotype Ad vectors to rodents will circumvent systemic and local host immunity elicited by the first vector administration (57, 72, 76). Thus, different-serotype Ad gene transfer vectors, or vectors with capsid components modified at specific capsid sites known to be targets for the anti-Ad neutralizing antibodies, might be used in the context of preexisting, serotype-specific anti-Ad immunity (1, 10, 33, 64, 95, 99, 113, 126). Second, several strategies to suppress humoral immune responses to Ad vectors, including immunotolerization by different routes, cytokine treatment, use of monoclonal antibodies, T-cell depletion strategies, macrophage depletion, thymectomy, and immunosuppressive drugs, have been evaluated in experimental animals (11, 15, 22, 24, 25, 27, 34, 35, 37, 38, 44, 47, 48, 51, 52, 58, 62, 66, 100, 101, 104, 110, 114, 123, 124, 127, 130, 131, 135, 136, 140); many of these strategies have proven successful in blunting humoral immune responses, permitting transgene expression after repetitive administration of Ad vectors (11, 15, 22, 44, 47, 48, 58, 62, 101, 110, 124, 127, 133, 135, 136). These strategies have yet to be evaluated in humans.

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