HUMAN GENE THERAPY 8:1263–1273 (July 1, 1997)
Mary Ann Liebert, Inc.

Anti-Vector Immunoglobulin Induced by Retroviral Vectors

JAMES E. McCORMACK, DARLENE MARTINEAU, NICHOLAS DePOLO, STEPHANIE MAIFERT, LEILA AKBARIAN, KAY TOWNSEND, WILL LEE, MIKE IRWIN, NANCY SAJJADI, DOUGLAS J. JOLLY, and JOHN WARNER[1]

ABSTRACT

Replication-incompetent retroviruses have been employed as gene therapy vectors in experimental settings for more than a decade. More recently, these vectors have been tested in the clinic as immunotherapeutic agents and anticancer agents. One potential problem with the use of such vectors is the possible development of immune responses directed against the vector particles themselves. Here, we examine immunoglobulin (Ig) responses specific for retroviral vectors derived from murine leukemia virus (MLV). Anti-MLV Ig is seen following intramuscular (i.m.) administration of retroviral vectors in mice, and in nonhuman primates; as expected, these responses are dependent upon the vector dose delivered. Furthermore, serum from vector-treated animals is capable of partially neutralizing vector-mediated transduction of target cells in an *in vitro* assay. Nevertheless, even in the presence of significant levels of anti-vector Ig *in vivo,* i.m. administration of retroviral vector is still capable of driving both Ig and cytotoxic T lymphocyte (CTL) responses specific for vector-encoded gene products. This work suggests that although retroviral vectors may readily induce immune responses directed against the vector particles themselves, such responses will not significantly affect the efficiency of these vectors in an immunotherapeutic protocol.

OVERVIEW SUMMARY

We have examined the immunoglobulin (Ig) response directed against retroviral vector particles in rodent and nonhuman primate models.In all animals examined to date, intramuscular (i.m.) administration of retroviral vector induces strong anti-vector Ig responses, in a dose-dependent fashion. Although such anti-vector Ig is capable of neutralizing vector in an *in vitro* assay, no adverse effect on vector efficacy was seen following repeated i.m. administration.

INTRODUCTION

REPLICATION-INCOMPETENT RETROVIRAL VECTORS have been used to mediate gene transfer for experimental purposes for more than 12 years. Since the first description of such vectors, it has been clear that the delivery of exogenous genetic material has great potential as a therapeutic tool, whether for the treatment of genetic disorders, or for delivery of foreign genes in immunotherapeutic regimens (Jolly and Warner, 1990).

Many animal studies, and the majority of clinical studies reported to date, have employed an *ex vivo* approach to gene delivery (Crystal, 1995), wherein cells are removed from an individual, transduced *in vitro,* and readministered. This approach has been useful in establishing the safety and potential for efficacy of gene transfer; however, such protocols are somewhat cumbersome for routine use in the clinic. It is hoped that techniques and vectors can be developed such that direct administration of vector will lead to the desired therapeutic effects. To that end, we have been testing a retroviral vector encoding the *env* and *rev* genes of human immunodeficiency virus type IIIB (HIV_{IIIB}) (Ziegner *et al.,* 1995) as an immunotherapeutic for HIV-infected patients; approximately 300 patients have been treated to date. Other groups are also testing direct delivery of retroviral vectors in clinical trials (Roth *et al.,* 1996).

If retroviral vectors are to be administered directly to pa-

Chiron Technologies, Center for Gene Therapy, San Diego, CA 92121.
[1]Present address: Inex Pharmaceuticals Corp., Vancouver, British Columbia V6P 6P2.

tients, it is important to characterize immune responses generated by the vector particles themselves, as these responses may adversely affect the course of therapy. Retroviral vector particles have physical characteristics similar to the retroviruses from which they are derived—in this case, Moloney murine leukemia virus (MLV). This type C retrovirus is approximately 100 nm in diameter; the major surface antigen, and primary target of neutralizing Ig, is the MLV envelope protein, gp70 (Coffin, 1990). Such a polyvalent antigen might be expected to elicit a strong vector-specific immunoglobulin (Ig) response. Ig responses directed against vector are of particular concern in cases where multiple courses of administration of retroviral vector are necessary; anti-vector Ig might significantly diminish or completely block the effects of vector "boosts."

Here we have characterized anti-vector Ig responses induced by intramuscular (i.m.) administration of MLV-based retroviral vectors in animal models. Significant levels of anti-vector Ig are induced by such treatment, both in mice and non-human primates. However, although such antibodies showed some ability to neutralize vector activity in an *in vitro* assay, they did not block vector activity *in vivo*. Animals with significant titers of anti-vector Ig were still capable of mounting immune responses against vector-encoded genes.

MATERIALS AND METHODS

Vector production

All retroviral vectors used in this study were produced by packaging cells derived from the DA cell line, which is derived from a dog osteosarcoma line (D-17; ATCC #CCL 183) expressing Moloney gag and pol, along with amphotropic env proteins. The HIV-IT(V) vector and the βgal(V) vector have been described previously (Irwin *et al.*, 1994; Ziegner *et al.*, 1995). The HB-IT(V) (a.k.a. 6A3) vector encodes a fusion protein joining the hepatitis B core protein (adw subtype) to the neomycin phosphotransferase protein; construction of this vector will be described in detail elsewhere (Townsend *et al.* 1997).

Culture supernatant containing retroviral vector was harvested from vector producer cell lines and concentrated, purified, and formulated to the indicated titers. Purified preparations were concentrated by tangential flow filtration and dialysis, and purified by size-exclusion chromatography; the final isotonic formulation buffer contains NaCl, human serum albumin (HSA), and lactose (Patent Application WO 95,10601). Vector titer was determined by serial dilution and colony-formation assay on HT 1080 indicator cells selected in G418-containing medium. All vector preparations used *in vivo* were assayed for the presence of replication-competent retrovirus (RCR) by S^+L^- assay (Miller and Buttimore, 1986); all preparations used scored negative by this assay.

Animals and treatments

Female BALB/c mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and housed under specific-pathogen-free conditions in our vivarium. Mice were injected with 0.1–0.2 ml of HIV-IT(V) vector i.m., distributed between two muscle sites, as indicated. Serum samples were collected at the indicated times, and stored at −80°C until analysis.

Rhesus macaques were maintained at the White Sands Research Center (Alamogordo, NM). Macaques were injected i.m. with a total of 2.0 ml vector distributed evenly between four muscle sites. Animals were injected with vector, and serum samples were drawn, processed and frozen at the indicated times.

Chimpanzees, chronically infected with hepatitis B virus, were maintained at the White Sands Research Center (Alamogordo, NM). Immunizations were performed with a total of 2.0 ml of vector evenly distributed between four muscle sites.

Reagents

Horseradish peroxidase (HRP)-conjugated goat anti-(macaque IgG) was obtained from Nordic Immunological Laboratories (Capistrano Beach, CA). Isotype-specific, affinity-purified HRP-conjugated goat anti-(mouse Ig) sera were from Southern Biotechnology Associates (Birmingham, AL). HRP-conjugated goat anti-(human IgG) was purchased from Tago (Burlingame, CA), and was used to detect chimpanzee Ig. TMB substrate was purchased from Bio-Rad (Hercules, CA).

ELISA buffers

Phosphate-buffered saline (PBS) was 10 × PBS prepared from pre-mixed salts (Amresco; Solon, OH). Blocking buffer was 2% teleost skin gelatin (Sigma, St. Louis, MO) in PBS containing 0.1% sodium azide. Wash buffer was 0.05% Tween-20 (Fisher Scientific; Pittsburgh, PA) in PBS. Dilution buffer was 1% bovine serum albumin (BSA; Sigma), 1% human serum albumin (Alpha Therapeutic Corp., Los Angeles, CA), 2.5% fetal calf serum (FCS; Irvine Scientific; Santa Ana, CA), 1% teleost skin gelatin, and 0.05% Tween-20 in PBS.

ELISA assays

Antigens were diluted in PBS containing 0.1% sodium azide; vector [βgal(V), suspended in a Tris buffer with no HSA] was used at a 1:10 dilution, whereas gp120 was used at 1 μg/ml. A total of 100 μl of antigen solution was added to wells of a polystyrene enzyme-linked immunosorbent assay (ELISA) plate (COSTAR #3591, Cambridge, MA), whereas wells to measure background binding received PBS/azide only. Plates were stored overnight at 4°C. In the morning, 100 μl of blocking buffer was added to each well; plates were stored at room temperature for 1 hr, and then washed with an automated plate washer (Bio-Tek Instruments; Winooski, VT). Test sera were diluted in dilution buffer, incubated for 1 hr at room temperature, and spun at 10,000 rpm for 5 min prior to plating. Serial dilutions were performed on the assay plate; all sera were bound to both antigen-coated and control, uncoated wells. After incubating for 5 hr at 37°C, plates were washed, and HRP-linked secondary reagents were added. Following a further hour at room temperature, plates were washed, and TMB substrate was added. The reaction was stopped by the addition of an equal volume of 1 N H_2SO_4, and absorbance was determined at 450 nm. Background readings were subtracted from test readings in all cases.

For studies of mouse sera, standard curves were generated on each plate by coating wells with purified goat anti-(mouse Ig κ chain) (Caltag, San Francisco, CA) overnight. While sera

were binding to assay wells, serial dilutions of mouse myeloma proteins (defined H chain isotypes; κ light chains; Sigma, St. Louis, MO) were incubated in anti-κ coated wells. Data from the same plate were quantitated relative to this standard curve using the SoftMax analysis package (Molecular Devices, Palo Alto, CA).

A pool of sera from 4 macaques that had received autologous vector-transduced bone marrow contaminated with replication-competent virus (Donahue *et al.,* 1992) was the kind gift of R.E. Donahue (National Heart, Lung and Blood Institute, Bethesda, MD). This was used as a positive control in analysis of macaque anti-vector Ig.

Western blots

Protein samples were run on 8–16% Tris-glycine polyacrylamide gels (NOVEX, San Diego, CA) under reducing conditions, and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Samples included: lysates from DA and/or D17 cells (1.5×10^5 cell equivalents/lane); retroviral vectors HIV-IT(V) and/or βgal(V) (20 μl of concentrated, purified, unformulated material; approximately 1–2 μg of total protein); HSA (5 μg per lane); and FCS (10 μl of a 1:5,000 dilution; ~1 μg total protein).

Membranes were immersed in a blocking reagent (Boehringer Mannheim, Indianapolis, IN) with 3% BSA added, then incubated overnight with test sera diluted 1:1,000 in blocking reagent. After washing in a solution of PBS/0.2% Tween 20, HRP-conjugated secondary antibodies were added. Goat anti-(mouse IgG) was from Jackson ImmunoResearch (West Grove, PA) and was used at a dilution of 1:15,000, whereas goat anti-(macaque IgG) from Nordic Immunolological Labs (Capistrano Beach, CA) was used at 1:2,000. One hour later, membranes were washed extensively. Signal was revealed by immersion for 1 min in the ECL chemiluminescent substrate (Amersham, Arlington Heights, IL) and exposure to Hyperfilm ECL (Amersham).

Cytotoxic T lymphocyte assays

Cytotoxic T lymphocyte (CTL) assays were performed essentially as described previously (Irwin *et al.,* 1994; Warner *et al.,* 1995). Spleens were collected from mice 21 days after immunization with HIV-IT(V) retroviral vector. Single-cell suspensions were prepared and restimulated *in vitro,* using irradiated stimulator cells expressing HIV_{IIIB} env and rev proteins, at an E/T ratio of 50:1. After 7 days, specific cytolytic activity was measured in a ^{51}Cr-release assay.

Vector neutralization assay

Serum samples were heat-inactivated for 30 min at 56°C prior to use in this assay. Equal volumes (20 μl) of serum and freshly thawed βgal(V) vector were mixed in sterile tubes, and incubated for 1 hr at 37°C. Following this incubation, samples were added to wells with HT1080 cells (human fibrosarcoma; ATCC CCL121) in medium containing 8 μg/ml Polybrene (Sigma). Forty-eight hours later, cells were lysed and β-galactosidase (β-Gal) activity was measured using the Galacto-Light Plus chemiluminescent substrate (TROPIX Inc., Bedford, MA). Light release was measured by luminometer (Analytical Luminescence Labs, San Diego, CA). Vector neutralization is calculated as the reduction of β-Gal activity, due to decreased transduction of HT1080 cells, relative to a mock-treated control, using the formula % neutralization = ([(control RLU − test RLU) ÷ control RLU] × 100%). A standard curve comprised of serial dilutions of untreated βgal(V) vector was included in each experiment; all sample readings shown here fall in the linear range of this assay.

RESULTS

Anti-vector Ig

Mouse: HIV-IT(V): To test for Ig directed against vector particles, an Ig capture ELISA assay was developed, using plate-bound vector particles as the capture antigen. The retroviral vector βgal(V) was used to coat plates in this assay. This vector carries the gene for β-Galactosidase, and is produced by a packaging cell line derived from DA, the same parental line used in the construction of other vector-packaging cell lines (VPCLs) used in this study. Briefly, DA is derived from the dog osteosarcoma line D17, and carries the Moloney MLV *gag* and *pol* genes and the 4070A amphotropic *env* gene on separate cistrons (Jolly, 1994).

Use of the βgal(V) vector in the assay offers two advantages: First, it can be used to assay anti-vector responses in animals treated with any vector produced by VPCLs derived from DA. Second, in the case of vectors that carry genes encoding cell-surface proteins [*e.g.,* HIV gp120 encoded by the HIV-IT(V) vector], the VPCLs will express and display this protein on their surface. It has been reported that nonviral cellular proteins can be found incorporated in retroviruses (see, for example, Suomalainen and Garoff, 1994; Arthur *et al.,* 1992); thus, it is possible that a protein such as gp120 might be incorporated at low levels in the envelope of the corresponding vector particles. In such a case, antibodies directed against the vector-encoded gene product could contribute to background noise in this assay; use of βgal(V) as antigen obviates this concern.

To study the development of anti-vector Ig following i.m. injection of retroviral vector, BALB/c mice were injected with either 4×10^6 cfu (HI) or 4×10^5 cfu (LO) of HIV-IT(V) vector as described in Materials and Methods. Sera were collected at regular intervals, and assayed for the presence of anti-vector Ig. Prior to vector treatment, no IgG that binds vector can be detected in the serum of BALB/c mice. As shown in Fig. 1, administration of HIV-IT(V) vector leads to the production of significant levels of anti-vector IgG_1. Not surprisingly, induction of anti-vector Ig is dose-dependent. Furthermore, a rapid and pronounced boosting effect is seen when animals are rechallenged with the same dose of vector 22 weeks after initial treatment (compare scales on Fig. 1 and inset).

IgG_1 is the most prevalent isotype seen among anti-vector immunoglobulins in this study. At week 24, following a vector boost, levels of anti-vector IgG_1 exceed 100 μg/ml in high-dose animals, and are roughly half this level in low-dose animals. Significant levels of other Ig isotypes are also present at this time. IgG_{2a} is second in abundance (5–10 μg/ml), whereas IgG_{2b} (1–2 μg/ml) and IgG_3 (0.2–2 μg/ml) are present at lower levels. Low but detectable levels of IgA specific for vector are

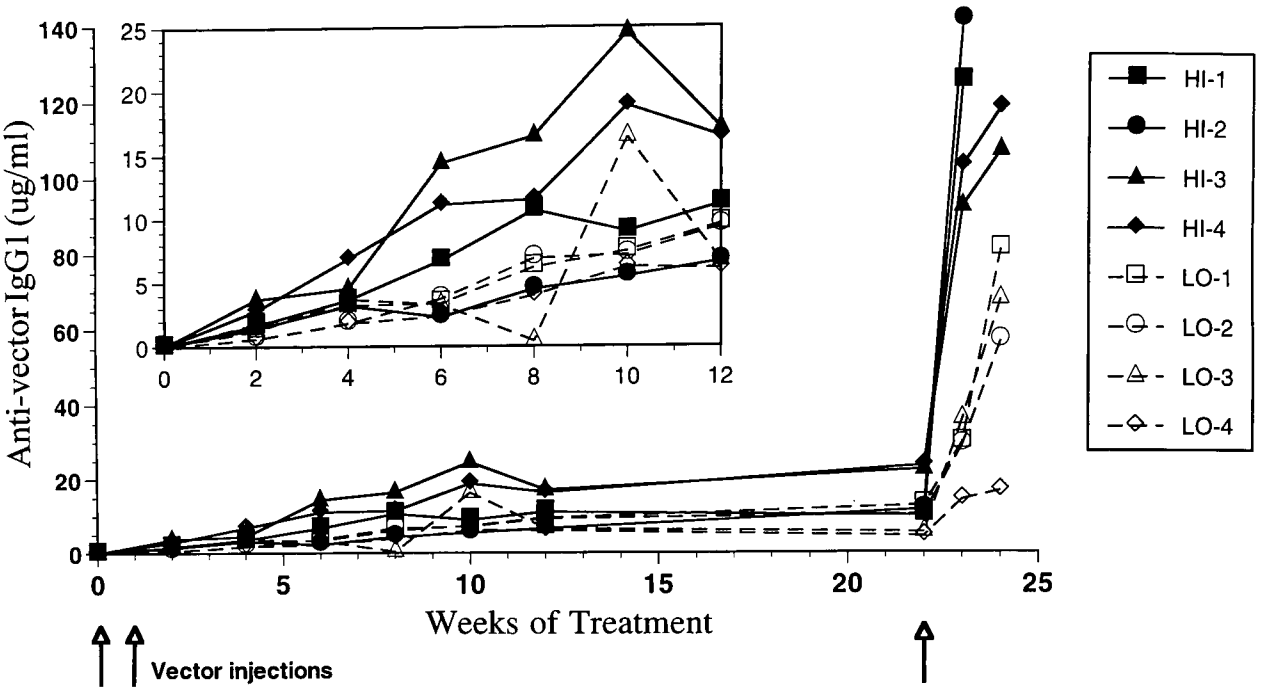

FIG. 1. Mice develop high titers of IgG_1 that bind retroviral vector following exposure to retrovector. BALB/c mice were injected with HIV-IT(V) vector, receiving a total of either 4×10^6 cfu (HI, closed symbols) or 4×10^5 cfu (LO, open symbols), as described in Materials and Methods. At the indicated times, serum samples were drawn. Samples were subjected to ELISA analysis and levels of IgG_1 specific for retroviral vector were quantitated as described. Inset: Expanded view of IgG_1 timecourse, weeks 0–12.

also present in serum. Vector specific IgM was present following initial exposure to vector, then declined, whereas vector-specific IgE was not detectable (data not shown).

As expected, animals receiving higher doses of vector generally display higher levels of anti-vector Ig; this is particularly notable following a boost. However, it must be noted that there is a wide range of variability in responses between individual animals, both in overall levels of Ig induced by vector exposure and in the distribution of Ig isotypes.

Non-Human Primates: HIV-IT(V) and HB-IT(V): In similar studies, the induction of anti-vector Ig responses was examined in rhesus macaques. In one study, animals were injected i.m. with HIV-IT(V) vector at either a high (2×10^8 cfu) or a low (2×10^7 cfu) dose, at weeks 0, 2, and 4; sera were collected at the indicated times, and analyzed for levels of anti-vector Ig (Fig. 2A). As in mice, an initial course of vector exposure leads to a rise in vector-specific IgG. Ig levels rise until 2–4 weeks after completion of the first course of injections, and then slowly drop. A boost at week 19 leads to a rapid rise in anti-vector IgG. Animals receiving higher doses of vector displayed uniformly higher levels of anti-vector IgG. The dashed line indicates the signal generated by a positive control sample at the same dilution. This is a pool of sera from macaques that were accidentally exposed to a replication-competent retrovirus, and that developed high titers of anti-MLV Ig (Donahue *et al.*, 1992).

In a separate study, macaques were treated with the HB-IT(V) vector (2.8×10^9 cfu) encoding the hepatitis B virus core protein. Injections were given at weeks 0, 4, 8, and 12, followed by a period of rest, and a boost at week 38. Although this course of treatment differs from the HIV-IT(V) study (Fig. 2A), the overall anti-vector response to HB-IT(V) is similar (Fig. 2B). Curiously, although the measured titer of this vector preparation is higher than that of the HIV-IT(V) vector, the Ig response induced is not as strong. (Compare signals of test sera to the positive control, represented by the dashed line, in Fig. 2 A and B.) It is unclear whether this difference due to the different course of administration (vector every 4 weeks vs. every 2 weeks), or due to differences in the vector preparations employed.

In a related study, 2 chimpanzees chronically infected with HBV were injected with the HB-IT(V) vector (2.8×10^9 cfu). Vector was administered at weeks 0, 2, 4, and 6, with a boost at week 28. Sera from these chimps were analyzed for the presence of anti-vector Ig. As in mice and macaques, an initial series of vector injections produces a gradual rise in vector-specific IgG over the course of treatment (data not shown). This activity peaks after treatment stops, and gradually declines. A subsequent boost at 28 weeks produces a rapid spike in vector specific IgG, which declines with time.

Immunoblot analysis

The ELISA assay employed above is a quick and sensitive means of detecting anti-vector Ig. However, the ELISA does not give information regarding the specific antigens being recognized. To address that question, test sera were used to probe protein blots as described in Materials and Methods. Representative results are shown in Fig. 3.

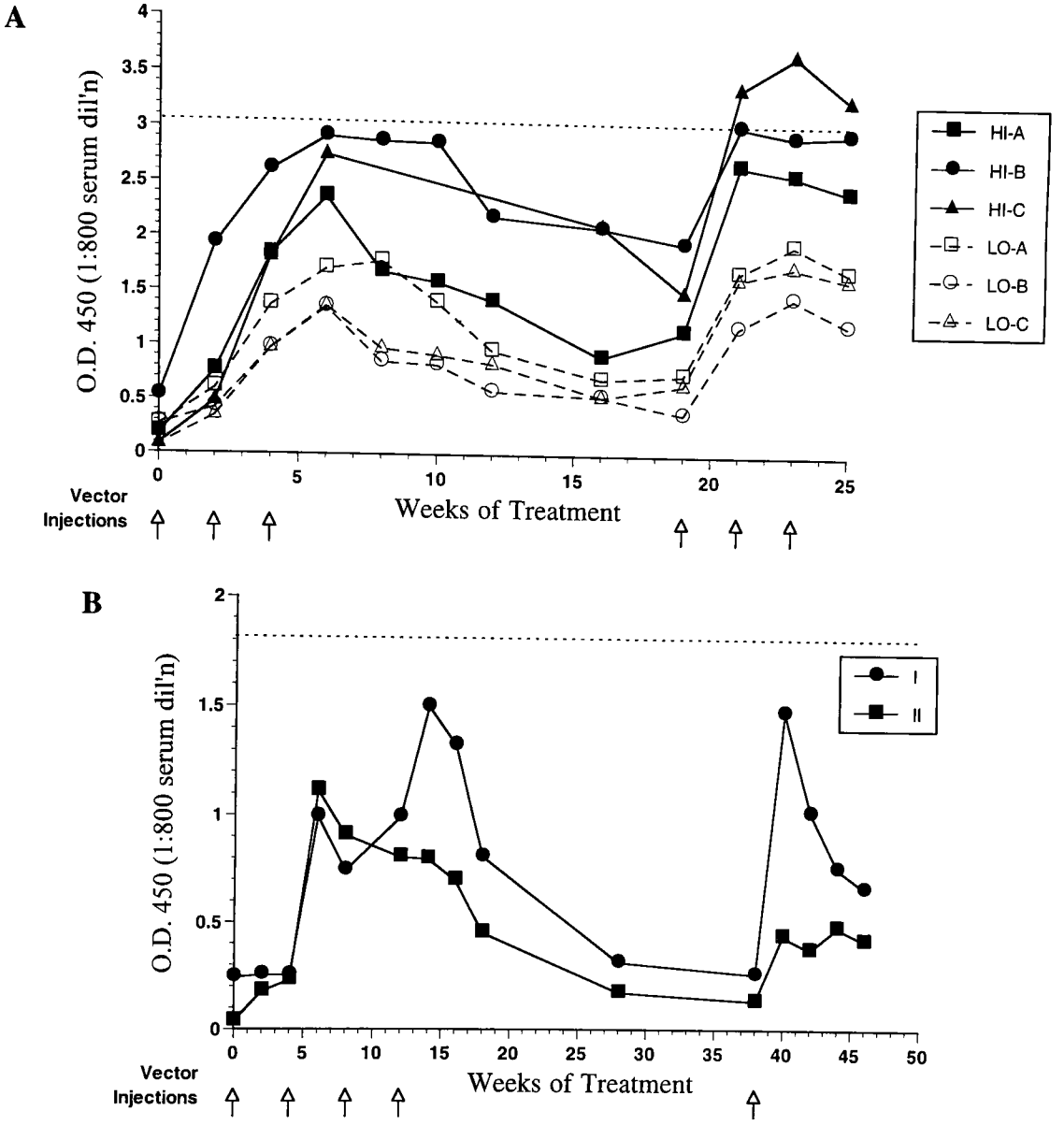

FIG. 2. Macaque anti-vector IgG: two studies. A. Rhesus macaques were injected i.m. at the indicated times with HIV-IT(V) vector, receiving either 2×10^8 cfu (HI, closed symbols) or 2×10^7 cfu (LO, open symbols), as described in Materials and Methods. IgG binding to retroviral vector was detected by ELISA, as described; data are plotted as OD_{450} at a 1:800 dilution of serum. The dotted line shows the signal generated by a positive control macaque serum at the same dilution. B. Two macaques were injected at the indicated times with HB-IT(V) vector (2.8×10^9 cfu) as described. IgG binding to retroviral vector was assayed and plotted as above.

Immunoblot analysis of post-treatment mouse sera reveals strong Ig responses directed against bands in the appropriate positions for both env and gag determinants. Figure 3A shows a protein blot probed with serum from a mouse treated with HIV-IT(V) vector (animal HI-1 in Fig. 1; 6-week timepoint). The first lane contains a lysate of DA cells, the parental packaging cell line which expresses the MLV *gag, pol,* and *env* genes. Clear bands are seen at 70 kD, corresponding to the env protein gp70, and 15 kD, which might be either the env anchor segment of gag p15. Fainter bands are visible at 80–90 kD, which may represent env or gag precursors (Weiss *et al.,* 1984). Strong binding to a band at the expected position for the virion core protein p30 is also seen. In lane 2, HIV-IT(V) vector was lysed in sample buffer and run on the gel; here bands at gp70, p30, and p15 predominate, whereas processing intermediates are not seen. Lane 3 contains FCS. This animal did not display anti-FCS activity after a primary treatment with vector, but such antibodies are present after a vector boost (data not shown). Lane 4 contains HSA, a component of the buffer in which vector is delivered. Strong Ig responses to HSA have been detected in all mice tested to date that have been exposed to vector suspended in formulation buffer containing HSA.

Figure 3B shows a similar blot probed with macaque serum (animal HI-C in Fig. 2A). Lane 1 is a lysate from D17, the dog

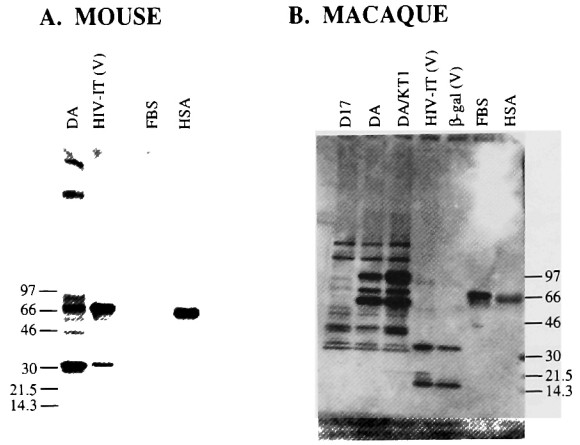

FIG. 3. Immunoblot analysis of anti-vector response. First, 8–16% polyacrylamide gels were loaded with the indicated samples under reducing conditions: lysates from DA, D17 cells (1.5×10^5 cell equivalents); retroviral vectors HIV-IT(V), βgal(V) (20 μl of concentrated, purified, unformulated material); HSA (0.5 μg); FCS (10 μl of a 1:5,000 diliution; ~0.1 μg total protein). Proteins were transferred to nylon membranes as described in Materials and Methods. Blots were probed with serum from (A) BALB/c mouse treated with HIV-IT(V) vector (Fig. 1, HI-1; 6-week timepoint), and (B) rhesus macaque treated with HIV-IT(V) vector (Fig. 2A, HI-C; 6-week timepoint).

osteosarcoma cell line from which the packaging cell lines employed here were derived. Several bands are visible here, indicating the presence of Ig reactive with dog proteins. However, the same bands are seen when parallel blots are probed with serum from naive macaques that have not been exposed to vector (data not shown). Thus, this activity is not induced, nor does it appear to be boosted, by exposure to dog proteins that might be present in the vector preparation. The origin of this cross-reactivity is unknown; however, we believe that the proteins detected here are dog proteins, and not retroviral proteins, as we have been unable to detect production of any retroviruses by D17 cells under any culture conditions, either by S^+L^- assay (Miller and Buttimore, 1986) or by electron microscopy. Further, Southern blot analysis of DNA from D17 cells does not reveal any cross-hybridization to MLV sequences (data not shown).

The next two lanes contain lysates of packaging cell lines—DA, the parental packaging cell, and DAKT1, the cell that produces HIV-IT(V) vector. Bands that presumably correspond to gag and/or env products are visible at 80–90 kD, 70 kD, and 50–60 kD; p15 and p30 bands are not apparent in these lysates, although an endogenous band present in D17 and its derivatives migrates at this position, and might obscure low levels of p30 reactivity. (We note that independently prepared cell lysates were used for the experiments of Fig. 4A, B. Cells in different stages of growth may display different proportions of viral proteins, which could explain the absence of p15 and p30 bands on this blot.) The following lanes contain proteins from preparations of HIV-IT(V) and βgal(V) vectors; the major bands seen here are at roughly 30 and 15 kD, suggesting that the major Ig response in this animal is directed against gag determinants. Little Ig specific for gp70 is seen at this point, although a weak response is seen after a boost with vector (data not shown). Binding is seen to HSA, as well as to a component of FCS, most likely BSA. Ig binding to HSA is also present in pre-

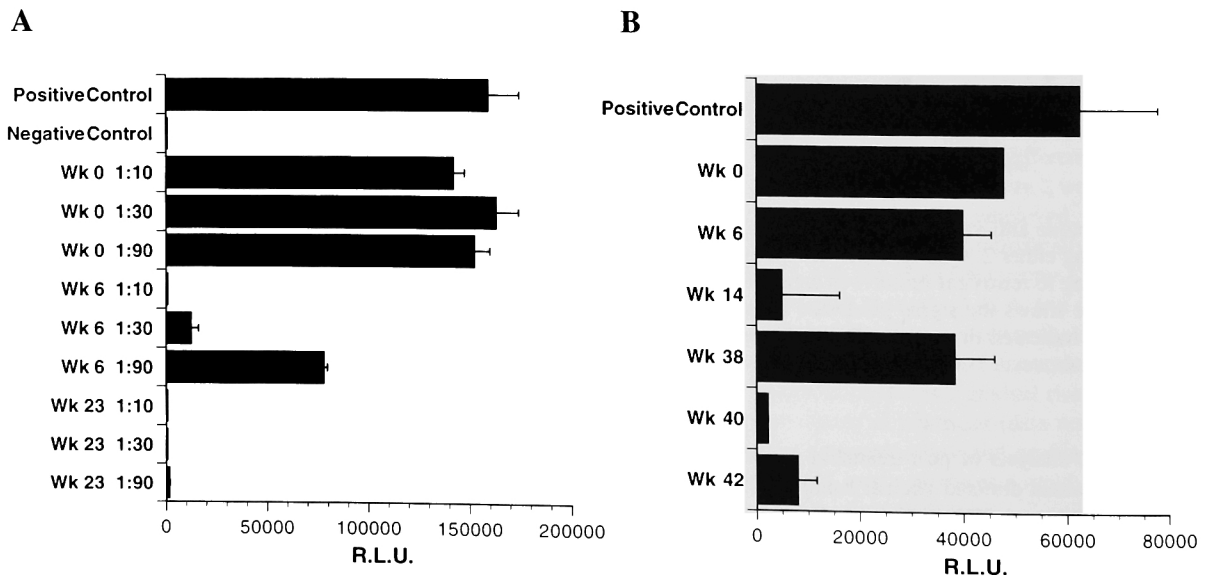

FIG. 4. Anti-vector IgG is capable of neutralizing retroviral vector activity. Aliquots of the retroviral vector DAβ-gal were preincubated with dilutions of macaque serum samples, then used to inoculate cell cultures, as described in Materials and Methods. Cells were lysed 2 days later, and β-gal activity was monitored using a chemiluminescent substrate. Data are plotted as mean relative light units emitted by each lysate, assayed in triplicate. A. Sera from a macaque treated with HIV-IT(V) (animal HI-C, Fig. 2A). Varying dilutions of sera from the indicated weeks—pretreatment, post-primary, and post-boost—were tested. Positive control, Mock-treated vector; negative control, no vector. B. Sera from a macaque treated with HB-IT(V) (Animal I, Fig. 2B). Sera from the indicated weeks were tested at 1:30 dilution. Positive control, Mock-treated vector.

treatment serum from this animal (data not shown). Sera from 3 other macaques in this study have been subjected to immunoblot analysis, with similar results—Ig binding patterns are consistent with a primary response predominantly directed against gag determinants, with anti-env responses revealed after a vector boost.

Vector neutralization

In Vitro: The appearance of Ig specific for gene therapy vectors could have adverse effects in therapeutic protocols calling for repeat administration of these vectors. Therefore, we asked whether the anti-vector Ig detected by ELISA was capable of neutralizing vector activity. To do this, an *in vitro* transduction assay was used, employing the βgal(V) retroviral vector. Vector particles were preincubated with serial dilutions of various control and test sera, then exposed to target cells. Vector-mediated gene transfer was assayed by measuring β-Gal activity in lysates of target cells, as described in Materials and Methods. Vector neutralization is revealed by a decrease in the vector-mediated transfer of β-Gal activity as the result of such preincubation.

The results of one such assay, performed on sera from a macaque treated with HIV-IT(V) vector (HI-C in Fig. 2A), are shown in Fig. 4A. Prior to exposure to HIV-IT(V), this animal's serum shows no vector neutralization activity. Six weeks following initial exposure to the retroviral vector, serum from this animal can block vector activity in this *in vitro* assay, with approximately 50% inhibition seen at a serum dilution of 1:90. At week 23, following a boost with HIV-IT(V), this animal's serum now shows an even stronger neutralizing activity.

Figure 4B shows results of a neutralization study on serum from a macaque that received HB-IT(V) vector (animal I in Fig. 2B). Neutralizing activity is seen in sera that display high levels of anti-vector Ig by ELISA (*cf.* weeks 14, 40, 42). Sera from other macaques, from both the HIV-IT(V) study of Fig. 2A and the HB-IT(V) study of Fig. 2B, have been similarly assayed for vector neutralizing ability. These data are compiled in Table 1, where they are presented as "% neutralization" as described in Materials and Methods. Varying degrees of vector neutralization have been revealed in all macaques tested to date, and the degree of neutralization appears to correlate with levels of anti-vector Ig. Sera from 2 chimpanzees exposed to HB-IT(V) vector were also assayed for neutralizing activity. Minimal neutralization of vector activity was seen in these studies (data not shown).

Effects on Vector In Vivo: The *in vitro* assay system described above reveals that anti-vector Ig generated in response to vector treatment has the capacity to inhibit vector-mediated transduction of target cells. However, it does not reveal whether anti-vector Ig actually inhibits retroviral vector action *in vivo,* in particular, under the conditions of i.m. injection of vector.

Observations from the above studies suggest that the anti-vector Ig generated in these animals is not sufficient to block vector activity completely. We have assayed for Ig directed against the products of genes encoded by the vector, in this case, gp120 of HIV. We have noted that a preliminary course of vector treatment, while inducing strong CTL responses against the vector-encoded gene product, does not induce significant levels of Ig against that gene product. However, a later boost with vector raises Ig specific for the gene of interest to detectable levels. Representative data illustrating this point are

TABLE 1. VECTOR NEUTRALIZATION VERSUS ANTI-VECTOR ELISA

Sample		*Percent neutralization (%)*	OD_{450}
HIV-IT(V) HI-C	Week 0	0.0	0.084
	Week 6	92.2	2.752
	Week 23	99.6	3.696
HIV-IT(V) LO-B	Week 0	5.1	0.267
	Week 8	17.9	0.851
	Week 23	26.0	1.462
HIV-IT(V) LO-C	Week 0	39.0	0.078
	Week 6	42.7	1.363
HB-IT(V) I	Week 0	23.6	0.242
	Week 6	36.2	0.992
	Week 14	92.2	1.499
	Week 38	38.7	0.258
	Week 40	96.6	1.477
	Week 42	87.4	1.015
HB-IT(V) II	Week 0	4.5	0.036
	Week 6	91.0	1.112
	Week 14	47.7	0.805
	Week 38	1.6	0.132
	Week 40	43.5	0.438
	Week 42	46.5	0.374

Rhesus macaques were treated with the indicated retroviral vectors as described in Fig. 2. Serum samples drawn at the indicated times were tested for anti-viral Ig by ELISA assay, and for vector neutralization as described. ELISA data are presented as OD_{450} at a serum dilution of 1:800. Neutralization data are presented as % inhibition of control signal ([(control RLU − test RLU) ÷ control RLU] × 100%) at a serum dilution of 1:30.

shown in Fig. 5. In Fig. 5A, mouse sera from the experiment of Fig. 1 were assayed for anti-gp120 IgG_1. Levels of gp120-specific Ig were near background levels following the initial course of treatment ("preboost," corresponding to Week 22 of Fig. 1), but rise to detectable, if low, levels following a second course of exposure to HIV-IT(V) vector. As the HIV-IT(V) vector encodes both the env and rev proteins of HIV_{IIIB}, we also assayed for the presence of Ig binding to rev_{IIIB}. As with env, little or no activity was seen following the initial course of vector treatment; a weak but detectable anti-rev response developed following the boost (data not shown).

Similar results are seen in macaques (Fig. 5B). Of the 6 animals in this study, only one (HI-B), which had received a high dose of vector, developed significant levels of anti-gp120 IgG following the first course of vector treatment. This animal and 3 others demonstrate significant boosting effects in response to a second exposure to vector. Serum from 1 animal (HI-A) showed anomalously high gp120 Ig binding in this assay prior

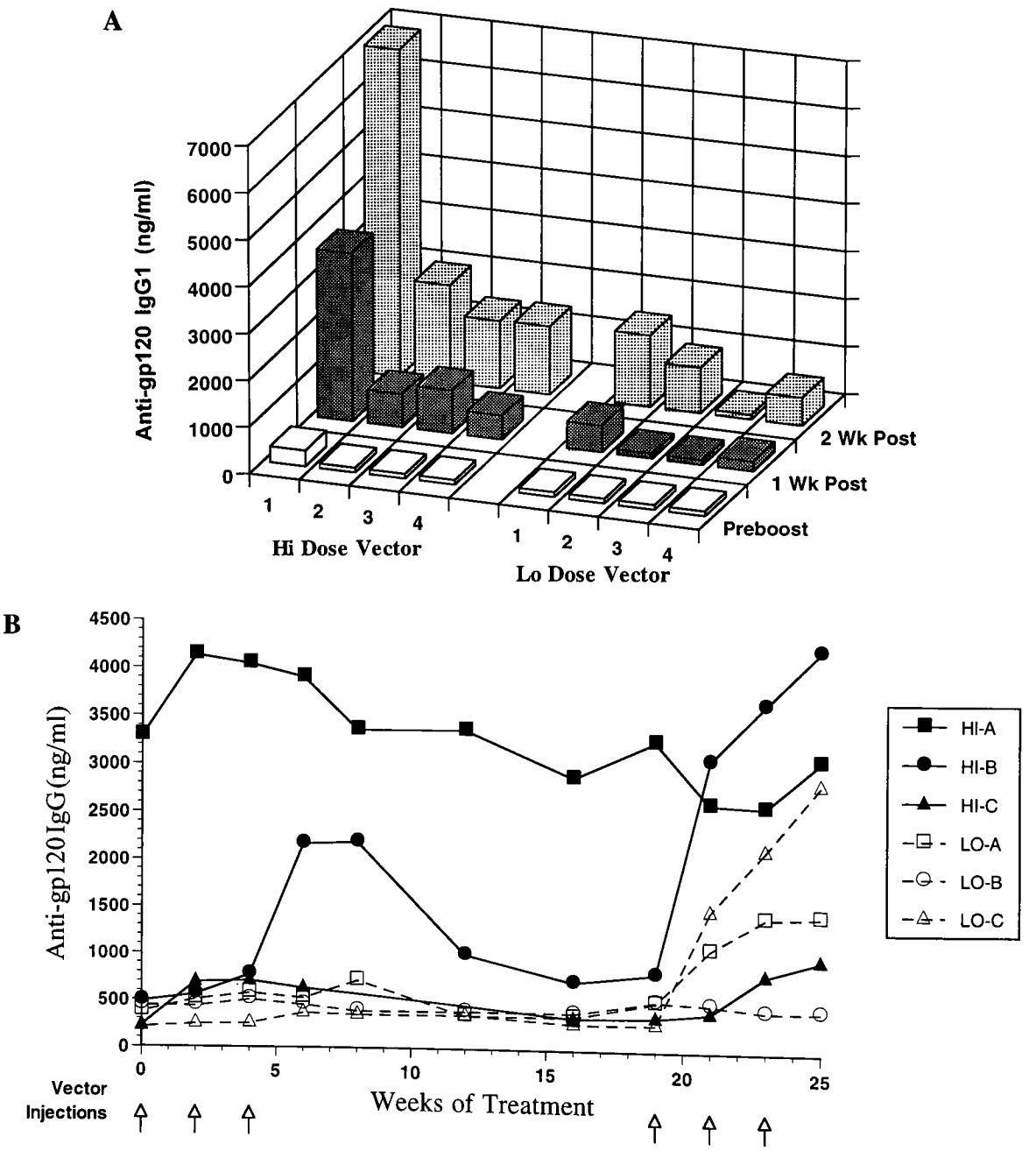

FIG. 5. Boost with vector boosts IgG response specific for vector-encoded HIV_{IIIB} gp120. A. Sera from the study depicted in Fig. 1 were assayed for the presence of IgG_1 specific for HIV gp120, as described in Materials and Methods. Preboost, 1 wk post, and 2 wk post correspond to weeks 22, 23, and 24 of Fig. 1. B. Sera from the study described in Fig. 2A were assayed for levels of IgG specific for HIV gp120, as described in Materials and Methods.

to vector administration; results from this animal are thus uninterpretable.

The fact that a second administration of vector can boost an immune response in animals displaying significant levels of anti-vector Ig indicate that this Ig is not sufficient to completely block vector activity *in vivo*. However, this result is somewhat inconclusive, as it may be that in this situation—an immune boost—a diminished level of vector activity is sufficient to produce the effects seen. Therefore, an experiment was designed to examine the effect of anti-vector Ig on the induction of a *de novo* immune response. BALB/c mice were pretreated with βgal(V) 4 and 5 weeks prior to HIV-IT(V) injection. This treatment led, as expected, to the development of anti-vector IgG at the time of HIV-IT(V) treatment (anti-vector IgG_1, 13–23 μg/ml; IgG_{2a}, 6.5–10.3 μg/ml). The development of anti-HIV CTL activity was monitored in both test animals and control animals (no βgal(V) pretreatment) as described in Materials and Methods. A significant inhibition of vector by pre-existing Ig should be reflected by diminished CTL activity.

The results of this experiment are shown in Fig. 6, which charts the lysis of target cells expressing the HIV env and rev antigens by effector cells from individual animals, at varying ratios of effectors to targets (E/T ratio). Strong CTL responses specific for env/rev are seen in all animals. Comparing HIV-IT(V)-induced CTL responses in naive animals (closed symbols; solid lines) with the responses seen in animals primed for anti-vector Ig (open symbols; dashed lines), no significant differences are seen. Thus, even though significant levels of anti-vector Ig are generated in response to a primary exposure to retroviral vector, this does not appear to impact the efficacy of vector subsequently administered i.m.

DISCUSSION

We report here the induction of anti-vector Ig following treatment of mice and non-human primates with retroviral vectors. Antigenically, retroviral vector particles are very similar, if not identical, to retroviral particles. The proteins expected to be most prevalent in the vector are the envelope glycoprotein, gp70; the transmembrane portion of the envelope, p15E; and the virion core protein, p30 (Weiss *et al.*, 1984). Antibodies binding each of these are seen in mice, macaques, and chimps exposed to retroviral vector (Fig. 3 and unpublished data).

Proteins other than those derived from the parent virus might also induce immune responses following vector administration. Proteins from the surface of the cell in which a virus of vector is produced can be incorporated at low levels in the budding virus or vector, rendering these proteins potential immunogens (Arthur *et al.*, 1992; Suomalainen and Garoff, 1994). Because the retroviral vectors used in these experiments were produced in a canine packaging cell line, they may carry small quantities of dog proteins on their surface. However, although some animals contain antibodies cross-reactive with dog proteins prior to vector treatment (Fig. 3), administration of vector has not been shown to increase these Ig responses.

The level of anti-vector Ig induced is, as expected, a function of vector dose. In both mouse and macaque, diluted vec-

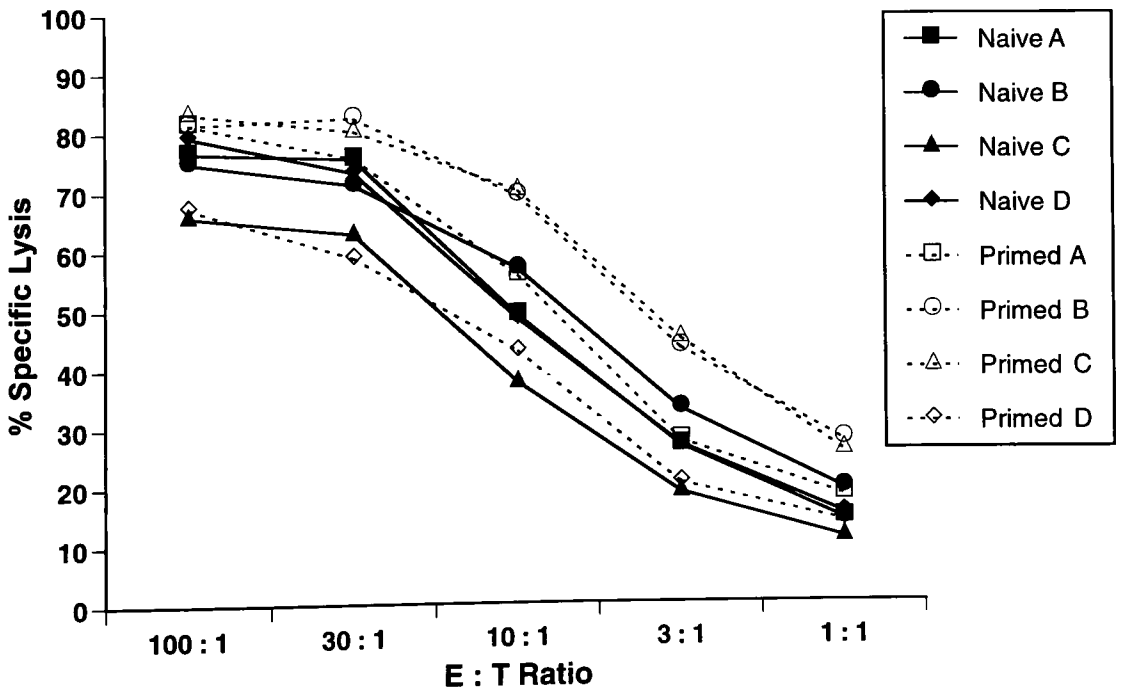

FIG. 6. Presence of anti-vector IgG does not block induction of CTL by retroviral vector. BALB/c mice were injected with βgal(V) vector several times, to induce anti-vector IgG. Animals were then immunized with HIV-IT(V) vector; naive control animals were immunized at the same time. Twenty-one days later, spleens were collected and assayed for env/rev-specific CTL activity as described in Materials and Methods. Data are plotted as the percentage specific target cell lysis seen at various effector/target (E/T) ratios. Closed symbols, solid lines: naive controls; open symbols, dashed lines: animals preprimed with βgal(V).

TABLE 2. VECTOR DOSING IN STUDIES (DIFFERENT SPECIES)

Mouse	3.3×10^6–3.3×10^8 cfu/kg	(0.1 ml @ 10^6–10^8 cfu/ml; 0.03 kg)
Macaque	2.5×10^6–2.5×10^8 cfu/kg	(2.0 ml @ 10^7–10^9 cfu/ml; 8.0 kg)
Chimp	3.3×10^5–3.3×10^7 cfu/kg	(2.0 ml @ 10^7–10^9 cfu/ml; 60 kg)
Human	2.5×10^4–2.5×10^5 cfu/kg	(2.0 ml @ 10^6–10^7 cfu/ml; 80 kg)

Retroviral vector doses per unit body mass for studies referred to in this manuscript. Mouse and human doses are for HIV-IT(V) vector. Chimpanzee doses are for HB-IT(V) vector. Macaque doses reflect separate studies involving both of these vectors.

tor induces a weaker Ig response than undiluted vector (Figs. 1 and 2A). In this light, we note that human subjects involved in a phase I clinical trial with HIV-IT(V) vector have demonstrated little or no anti-vector Ig (Martineau *et al.*, 1997). The doses per unit body mass delivered in this study are much lower than those used in animal studies (Table 2).

The immunogenicity of a vector preparation (with regard to anti-vector Ig development) may be enhanced by the presence of inactive vector particles in purified vector preparations. Preliminary studies employing electron microscopy to compare vector particle counts with measured titers, as well as studies comparing titers with net amounts of p30 protein, suggest that the ratio of total particles to active titer varies between different vector preparations. In this light, note that the levels of anti-vector Ig induced in macaques by HB-IT(V) vector are lower than Ig induced by HIV-IT(V) vector (Fig. 2). This is true despite the fact that the reported titer for the HB-IT(V) vector preparation used here is 10-fold higher than the HIV-IT(V) titer. In part, the difference in Ig induction by these two vectors may be due to differences in immunization schedules. However, it may also be that the ratio of inactive to active vector particles is higher in the HIV-IT(V) preparation. Better assays for particle count versus titer are needed to resolve this issue.

A factor that might contribute to anti-vector Ig responses in the macaque are antibodies that recognize the carbohydrate epitope Galα1-3Galβ1-4GlcNAc-R (α-galactosyl). This epitope is found on proteins from most mammalian species, but is not present in Old World monkeys or humans; these species have natural antibodies that bind this epitope (Galili, 1993). Anti-α-galactosyl Ig may account for some of the background binding to canine proteins seen in serum from unimmunized macaques; however, if this is true, it suggests that the glycosylation of MLV proteins by this packaging cell line does not include the α-galactosyl epitope, because bands corresponding to MLV gene products are only revealed by post-immunization sera. It is not clear whether anti-α-galactosyl antibodies adversely affect the function of these retroviral vectors *in vivo*; work is in progress to address this issue.

Another factor that may influence immune responses to vector in animal models is the presence of HSA in the vector formulation buffer. Ig binding HSA has been detected in all treated animals assayed for this activity (see, for example, Fig. 3); such reactivity is not expected to arise in human patients. However, the presence of this antigen does not appear to affect the development of anti-vector Ig. In one study, mice were treated with vector in formulation buffer containing either human serum albumin, mouse serum albumin, or no additional protein. Anti-vector responses were comparable in all cases, both after initial treatment and following a boost at 8 weeks (J.E.M. and L.A., unpublished observations).

It has been shown that the anti-vector Ig developed by animals in these studies has the capacity to neutralize vector in an *in vitro* assay (Fig. 4). The development of Ig with the ability to neutralize vector is of concern for therapeutic applications where multiple administrations of vector may be necessary. The potential impact of immune responses directed against retroviral vectors is underscored by recently published studies using adenoviral vectors. Adenoviral vectors have been shown to induce both cellular and humoral responses directed against adenoviral determinants; these responses have severely diminished the efficacy of subsequent boosts. This has been shown in studies with portal vein injection of vector into rabbits (Kozarsky *et al.*, 1994), intracardiac injection in rats (Kass-Eisler *et al.*, 1994), tail vein injection in mice (Barr *et al.*, 1995), intranasal administration in cotton rats (Yei *et al.*, 1994), and pulmonary delivery in mice (Yang *et al.*, 1995) and rhesus macaques (Kaplan *et al.*, 1996).

In the studies presented here, anti-vector Ig did not significantly impact upon vector-mediated induction or boosting of immune responses *in vivo* (*cf.* Figs. 5 and 6). However, the potential impact of such anti-vector Ig will vary, depending upon the route of administration. In the studies presented here, vector was administered via i.m. injection. This route of delivery should result in a high local concentration of vector particles, in a region of relatively low serum protein concentration. If the ability of vector to induce responses against the vector-encoded gene product is viewed as a competition between the processes of vector-mediated transduction of antigen-presenting cells and the inactivation of vector by Ig, complement, and other processes, then intramuscular injection would seem to tilt the balance in favor of vector-mediated transduction. Other routes of administration may result in different outcomes; for example, if administered intravenously (i.v.), vector will be rapidly diluted in a compartment containing high levels of Ig, and vector-neutralizing effects may be overriding. We note that although data presented here involve animals that have received i.m. injections of vector, anti-vector responses have also been observed in both rabbits and dogs following i.v. administration of vector (J.E.M., unpublished observations). Further study is necessary to determine whether this Ig interferes with subsequent i.v. vector delivery.

ACKNOWLEDGMENTS

The authors would like to thank Tammi Howard and Darci Knapp for animal care assistance; Carlos Ibañez and "Team

Vector" for retroviral vector production; Ann Beever for assisting with figures; and Wolfgang Klump for review of the manuscript.

REFERENCES

ARTHUR, L.O., BESS, J.W., SOWDER, R.C., BENVENISTE, R.E., MANN, D.L., CHERMANN, J.-C., and HENDERSON, L.E. (1992). Cellular proteins bound to immunodeficiency viruses: Implications for pathogenesis and vaccines. Science **258,** 1935–1938.

BARR, D., TUBB, J., FERGUSON, D., SCARIA, A., LIEBER, A., WILSON, C., PERKINS, J., and KAY, M.A. (1995). Strain related variations in adenovirally mediated transgene expression from mouse hepatocytes in vivo: Comparisons between immunocompetent and immunodeficient strains. Gene Ther. **2,** 151–155.

COFFIN, J.M. (1990). Retroviridae and their replication. In *Virology.* B.N. Fields, D.M. Knipe, R.M. CHANOCK, M.S. HIRSCH, J.L. MELNICK, T.P. MONATH, and B. ROIZMAN, eds. (Raven Press, New York).

CRYSTAL, R.G. (1995). Transfer of genes to humans: Early lessons and obstacles to success. Science **270,** 404–410.

DONAHUE, R.E., KESSLER, S.W., BODINE, D., MCDONAGH, K., DUNBAR, C., GOODMAN, S., AGRICOLA, B., BYRNE, E., RAFFELD, M., MOEN, R., BACHER, J., ZSEBO, D.M., and NIENHUIS, A.W. (1992). Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer. J. Exp. Med. **176,** 1125–1135.

GALILI, U. (1993). Interaction of the natural anti-Gal antibody with α-galactosyl epitopes: A major obstacle for xenotransplantation in humans. Immunol. Today **14,** 480–482.

IRWIN, M.J., LAUBE, L.S., LEE, V., AUSTIN, M., CHADA, S., ANDERSON, C.-G., TOWNSEND, K., JOLLY, D.J., and WARNER, J.F. (1994). Direct Injection of a recombinant retroviral vector induces human immunodeficiency virus-specific immune responses in mice and nonhuman primates. J. Virol. **68,** 5036–5044.

JOLLY, D. (1994). Viral vector systems for gene therapy. Cancer Gene Ther. **1,** 51–64.

JOLLY, D.J., and WARNER, J.F. (1990). Retroviral vectors as vaccines and immunotherapeutics. Sem. Immunol. **2,** 329–339.

KAPLAN, J.M., ST. GEORGE, J.A., PENNINGTON, S.E., KEYES, L.D., JOHNSON, R.P., WADSWORTH, S.C., and SMITH, A.E. (1996). Humoral and cellular immune responses of nonhuman primates to long-term repeated lung exposure to Ad2/CFTR-2. Gene Ther. **3,** 117–127.

KASS-EISLER, A., FALCK-PEDERSEN, E., ELFENBEIN, D.H., ALVIRA, M. BUTTRICK, P.M., and LEINWAND, L.A. (1994). The impact of developmental stage, route of administration and the immune system on adenovirus-mediated gene transfer. Gene Ther. **1,** 395–402.

KOZARSKY, K.F., MCKINLEY, D.R., AUSTIN, L.L., RAPER, S.E., STRATFORD-PERRICAUDET, L.D., and WILSON, J.M. (1994). *In vivo* correction of low density lipoprotein receptor deficiency in the Watanabe heritable hyperlipidemic rabbit with recombinant adenoviruses. J. Biol. Chem. **269,** 13695–13702.

MARTINEAU, D., KLUMP, W.W., MCCORMACK, J.E., DEPOLO, N.J., KAMANTIGUE, E., PETROWSKI, M., HANLON, J., JOLLY, D.J., MENTO, S.J., and SAJJADI, N. (1997). Evaluation of PCR and ELISA assays for screening clinical trial subjects for replication competent retroviruses. Hum. Gene Ther. **8,** 1231–1241.

MILLER, A.D., and BUTTIMORE, C. (1986). Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol. **6,** 2859–2902.

ROTH, J.A., NGUYEN, D., LAWRENCE, D.D., KEMP, B.L., CARRASCO, C.H., FERSON, D.Z., HONG, W.K., KOMAKI, R., LEE, J.J., NESBITT, J.C., PISTERS, K.M.W., PUTNAM, J.B., SCHEA, R., SHIN, D.M., WALSH, G.L., DOLORMENTE, M.M., HAN, C.-I., MARTIN, F.D., YEN, N., XU, K., STEPHENS, L.C., MCDONNELL, T.J., MUKHOPADHYAY, T., and CAI, D. (1996). Retrovirus-mediated wild-type *p53* gene transfer to tumors of patients with lung cancer. Nature Med. **2,** 985–991.

SUOMALAINEN, M., and GAROFF, H. (1994). Incorporation of homologous and heterologous proteins into the envelope of Moloney murine leukemia virus. J. Virol. **68,** 4879–4889.

TOWNSEND, K., SÄLLBERG, M., O'DEA, J., BANKS, T., DRIVER, D., SAUTER, S., CHANG, S.M., JOLLY, D.J., MENTO, S.J., MILICH, D.R., and LEE, W.T. (1997). Characterization of $CD8^+$ T-lymphocyte responses after genetic immunization with retrovirus vectors expressing different forms of the hepatitis B virus core and e antigens. J. Virol. **71,** 3365–3374.

WARNER, J.F., JOLLY, D., MENTO, S., GALPIN, J., HAUBRICH, R., and MERRITT, J. (1995). Retroviral vectors for HIV immunotherapy. Ann. N.Y. Acad. Sci. **772,** 105–116.

WEISS, R., TEICH, N., VARMUS, H., and COFFIN, J. (1984). *RNA Tumor Viruses—Molecular Biology of Tumor Viruses,* 2nd ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

YANG, Y., LI, Q., ERTL, H.C.J., and WILSON, J.M. (1995). Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J. Virol. **69,** 2004–2015.

YEI, S., MITTEREDER, N., TANG, K., O'SULLIVAN, C., and TRAPNELL, B.C. (1994). Adenovirus-mediated gene transfer for cystic fibrosis: Quantitative evaluation of repeated in vivo vector administration to the lung. Gene Ther. **1,** 192–200.

ZIEGNER, U.H.M., PETERS, G., JOLLY, D.J., MENTO, S.J., GALPIN, J., PRUSSAK, C.E., BARBER, J.R., HARTNETT, D.E., BOHART, C., KLUMP, W., SAJJADI, N., MERCHANT, B., and WARNER, J.F. (1995). Cytotoxic T-lymphocyte induction in asymptomatic HIV-1-infected patients immunized with Retrovector®-transduced autologous fibroblasts expressing HIV-1_{IIIB} Env/Rev proteins. AIDS **9,** 43–50.

Address reprint requests to:
Dr. James E. McCormack
Chiron Technologies
Center for Gene Therapy
11055 Roselle Street
San Diego, CA 92111

Received for publication December 20, 1996; accepted after revision April 25, 1997.