

Adenoviruses with an RGD-4C modification of the fiber knob elicit a neutralizing antibody response but continue to allow enhanced gene delivery

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Abstract

Objective. The purpose of this study was to investigate the effect of preexisting neutralizing antibody (NABs) in naive mice and the effect of induced NABs in mice immunized with either an RGD or nonmodified Ad5 vector on the transduction efficiency of adenoviral vectors.

Methods. BALB/c mice were immunized with Ad5LucRGD, with the unmodified Ad5Luc1, or with Opti-MEM intraperitoneally (ip) from one to three times. Sera were collected on day 27 and serially diluted to block Ad5Luc1 or Ad5LucRGD prior to infection of SKOV3.ip1 human ovarian carcinoma cells with these same vectors. Forty-eight hours post Ad infection, a luciferase assay was performed to determine the titer of NABs.

Results. Luciferase assay data showed that the gene transfer efficacy of Ad5LucRGD was 1.56-fold higher than Ad5Luc1 in the presence of serum from naive mice. In the presence of serum from Ad5Luc1-challenged mice, the transduction efficiency of Ad5LucRGD was 3.27-fold higher (single challenge) and 4.2-fold higher (triple challenge) than Ad5Luc1. In the presence of serum from Ad5LucRGD-challenged mice, the transduction efficiency of Ad5LucRGD was 2.24-fold higher (single challenge) and 2.53-fold higher (triple challenge) than Ad5Luc1.

Conclusion. The RGD-modified human Ad vectors appear to be less recognizable than unmodified Ad to preexisting NABs in mouse models. RGD-modified Ad vectors also appear to elicit a relatively lower level of NABs that may also contribute to the higher gene

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transduction efficiency of these modified vectors. Therefore, RGD-modified Ad vectors may be reagents of clinical utility in the context of preformed anti-Ad immunity and in the setting of repetitive dosing.
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Introduction

Adenoviruses (Ad) are among the most widely used gene delivery systems in current human gene therapy clinical trials [1]. To achieve the most beneficial therapeutic index, an ideal Ad vector-based gene therapy relies upon low toxicity and high transduction efficiency. Despite considerable advances in improving transduction efficiency and in prolonging transgene expression, the immunogenicity of Ad remains a major concern. Additionally, systemic immunity against the Ad vector is a fundamental obstacle for translation of gene therapy from preclinical to clinical utility. Since neutralizing antibodies (NABs) against Ad preexist in about half of human beings [2,3], the inflammatory response and NABs elicited by administration of Ad vector are potentially harmful to recipients and interfere with gene delivery by elimination of the vector. In an attempt to reduce the immunogenic properties of Ad vector, suppression of a recipient's immune response by several approaches [4] has been investigated.

Other investigators have focused on modifying Ad itself in an effort to overcome immunogenicity; examples of such modified Ad vectors include gutless Ad vectors, which are deleted of all genes encoding viral proteins [5–9], and attenuated Ad vectors, which have key early genes sequentially removed [10]. Packaging the viral genome into different subtypes of viruses has also been attempted [11–13]. Enthusiasm has also been generated by genetically engineering viral capsid protein to modify Ad tropism and thereby achieve a reduced immune response and more specific enhanced cell targeting [14–17]. As the knob domain of the fiber is the binding site for Ad5 to the primary cellular receptor the Coxsackievirus–Adenovirus receptor (CAR) [18], it is a major determinant of C type adenoviral tropism.

Viral tropism has been manipulated by a variety of approaches to accomplish retargeting goals [15–19]. An Ad5 vector with an RGD modification in the HI loop of the fiber knob was developed by our group [20,21] to enhance the infectivity of Ad5 vector in CAR-negative (or low) cell lines and primary ovarian cancer cells by redirecting the vector to bind to α_v integrin receptors [20,21]. Utilizing this approach, enhanced gene transduction was accomplished in ovarian cell lines and primary cells when compared to that achieved with nonmodified Ad [20,21]. Additional studies have demonstrated significant anti-tumor activity of RGD therapeutic vectors in murine models of ovarian cancer [22]. For in vivo and clinical application, understanding the antigenic

properties of RGD-modified Ad5 vectors is essential. In this study, the NAB profile was evaluated in nonimmunized mice and in mice immunized by either the RGD-modified vector or the unmodified parent. The effect of NABs from these sources on gene transfer efficiency of these same vectors was also evaluated.

Materials and methods

Cell line

The human ovarian cancer cell line SKOV.ip1 was obtained from Janet Price (M.D. Anderson Cancer Center, Houston, TX). The transformed human embryo kidney 293 cell line was purchased from Microbix (Toronto, Ontario, Canada). The cell lines were maintained in Dulbecco's Modified Eagle's medium/F12 (Mediatech, Herndon, VA) supplemented with L-glutamine (2 nM), penicillin (100 IU/ml), streptomycin (25 μ g/ml), and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) at 37°C in a humidified, 5% CO₂ atmosphere.

Adenoviral constructs

E1-deleted Ad5Luc1 encoding firefly luciferase under the control of the CMV promoter has been described [18]. The Ad5LucRGD containing the recombinant fiber RGD protein in the HI loop and expressing the firefly luciferase has also been previously reported [20]. Both viruses were propagated on 293 cells and were purified by a standard protocol [20]. The virus particle titer (vp) was determined by spectrophotometry at 260 nm, and the functional titer was determined with TCID50.

Mice

Twenty-five female BALB/c syngeneic mice at 4–6 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). Mice were kept under pathogen-free conditions according to the American Association for Accreditation of Laboratory Animal Care guidelines. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Generation of Nabs

For immunization of animals, the 25 mice were divided into five groups of five mice each. One group of mice

received Opti-MEM ip as control. Two groups of mice were immunized ip by Ad5Luc1 either with a single 9×10^5 vp injection on day 0 or sequentially on days 0, 3, and 6. Two groups of mice were immunized ip by Ad5LucRGD with 9×10^5 vp on day 0 or sequentially on days 0, 3, and 6, respectively. On day 27, whole blood from the mice was collected by intracardiac aspiration, and the sera were separated from whole blood by brief centrifugation and stored in -20°C for in vitro viral blocking.

Determination of effect of Nabs on gene transfer

To determine the effect of NAb on gene transfer efficiency, a luciferase assay was used to indirectly evaluate the titration of Nabs. A luciferase value (RLU) at a given dilution was converted into a functional titer of NAb at that dilution. A lower luciferase reading (RLU) reflects a higher level of NAb in immunized sera that have a greater blocking effect on preinfected adenoviral vectors and result in lower transduction efficiency and vice versa. Specifically, a 4-fold dilution series of serum from naive and immunized mice was prepared in Opti-MEM. SKOV3.ip1 cells were seeded into 96-well plates in quadruplicate (1×10^5 cells/well) and cultured overnight before viral infection [3]. Ad5Luc1 and Ad5LucRGD (100 vp/cell) were each mixed with diluted serum samples at room temperature for 30 min. Then, the mix was added to the cells and infection was allowed to proceed. Two hours later, infection medium was replaced by uninfected growth medium. After 48 h, luciferase expression was measured by the Luciferase Assay System (Promega, Madison, WI) utilizing an Orion Micro plate Luminometer (Berthold, Pforzheim, Germany) on CulturPlate™ -96 (Research Parkway, Meriden, CT) according to the manufacturer's instructions. The mixture was measured for 5 s/well and the data were normalized for protein concentration. The data were statistically analyzed by a paired Student's *t* test.

Results

Effect of preexisting Nabs on gene transfer

In this report, the two nonreplicative vectors, Ad5Luc1 and Ad5LucRGD, were compared with regard to preexisting NAb in naive mice. Specifically, all mice had a low but measurable titer at 1:1, 1:8, and 1:32 dilutions (Fig. 1). The overall inhibitory effect of Ad5Luc1-mediated luciferase expression by preexisting NAb was significantly higher (1.56-fold) than the Ad5LucRGD-mediated luciferase expression ($P = 0.04$) (Fig. 1).

Effect of generated Nabs on gene transfer

We compared the titers of anti-Ad5Luc1 and anti-Ad5LucRGD NAb of mice after single and triple immunization by Ad5Luc1 and Ad5LucRGD, respectively. Blocked by single challenged anti-Ad5Luc1 sera, the Ad5Luc1-mediated luciferase expression was overall significantly lower (3.27-fold) than with Ad5LucRGD (Fig. 2). A difference between the two vector-mediated luciferase levels was found in most dilutions excluding 1:512 ($P = 0.02$) (Fig. 2). Neutralized by triple-challenged anti-Ad5Luc1 sera, the Ad5Luc1-mediated luciferase expression was also significantly lower (4.21-fold) than with Ad5LucRGD (Fig. 3). A difference between the two vector-mediated transgene expressions was consistently found at all dilutions. The 1:32 dilution held the highest disparity (up to 11.16-fold) ($P = 0.0043$) (Fig. 3). Interestingly, higher gene transduction was noted in cells exposed to sera from mice immunized by either single or triple Ad5Luc RGD challenge when compared to cells exposed to sera from Ad5Luc1-challenged mice.

Neutralized by single-challenged anti-Ad5LucRGD sera, the Ad5Luc1-mediated luciferase expression averaged 2.24-

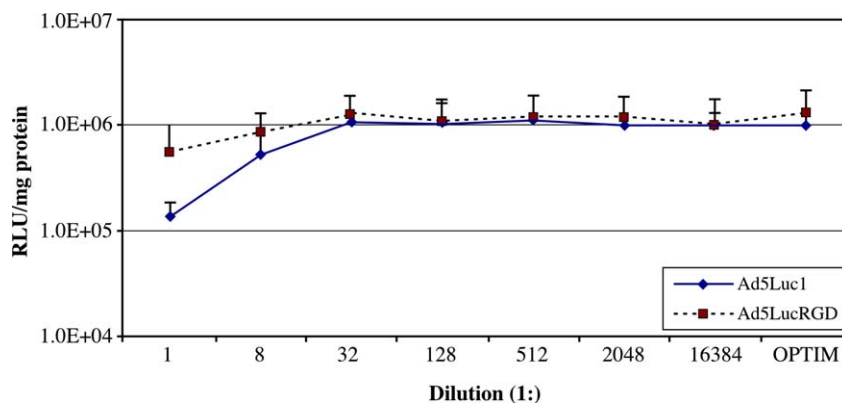


Fig. 1. Titration of preexistent inhibitors against Ad5Luc1 and Ad5LucRGD in mice sera. Opti-MEM was injected into mice ($n = 5$) three times. Collected sera on day 27 were employed to block Ad5Luc1 and Ad5LucRGD before infection of SKOV3.ip1 cells. A luciferase assay was carried out 48 h post-infection. Data represent the mean of quadruplicates (*t*-test, $P = 0.04$).

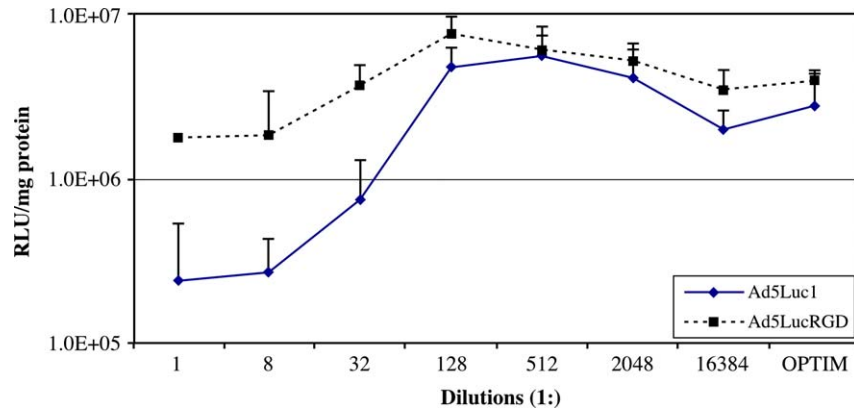


Fig. 2. One immunization with Ad5, and neutralization of Ad5RGD and Ad5. Mice ($n = 5$) were challenged once by Ad5Luc1. Collected sera on day 27 were employed to block Ad5Luc1 and Ad5LucRGD before infection of SKOV3 ip1 cells. A luciferase assay was carried out 48 h post-infection. Data represent the mean of quadruplicates (t -test, $P = 0.02$).

fold lower than Ad5LucRGD (Fig. 4). This approaches, but does not reach, statistical significance ($P = 0.06$). However, with triple challenged anti-Ad5LucRGD blocking sera, the Ad5Luc1-mediated luciferase expression was overall significantly lower (2.53-fold) than with Ad5LucRGD (Fig. 5). A statistical difference between the two vector-mediated transgene expressions was consistently found at all dilutions. The 1:128 dilution held the highest disparity [up to 4.96-fold] ($P = 0.0069$) (Fig. 5).

In addition, we compared one challenge by Ad5Luc1 (from Fig. 2) with Ad5LucRGD (from Fig. 4). Like Ad5Luc1, Ad5LucRGD also provoked a host humoral immune response and induced NAb, which neutralized the Ad5LucRGD and gave rise to decreased transgene expression (Fig. 6). However, the titer of anti-Ad5LucRGD NAb from one challenge of Ad5LucRGD was higher than the titer of anti-Ad5Luc1 from one challenge of Ad5Luc1 ($P < 0.05$) (Fig. 6). A comparison between a triple challenge by Ad5Luc1 (from Fig. 3) and that by Ad5LucRGD (from Fig. 5) shows no significant difference between the two vectors with three challenges ($P > 0.05$) (Fig. 7).

Discussion

Most people have preexistent NAb to different serotypes of Ad [23,24]. In the context of gene therapy, NAb can be induced by different administration routes as a result of a strong systemic immune response to Ad vector [25]. NAb may decrease efficiency of gene transduction [26]. Ad5LucRGD is a tropism-modified vector created by incorporation of an Arg–Gly–Asp sequence into the HI loop of the knob. Previous studies demonstrated that enhancement of infectivity and an expanded infection spectrum were achieved in human ovarian cancer cell lines and in primary human ovarian cancer cells [20,21]. An RGD-modified vector coding for thymidine kinase and a somatostatin receptor imaging moiety [27] and an RGD-modified conditionally replicative adenovirus are being developed for clinical trials, where the agents will be delivered intraperitoneally. Given the recombinant nature of the RGD-modified fiber, knowledge of the antigenic profile of this vector is fundamentally important. Indeed, we have seen higher rates of transfection with RGD-modified viruses. Our experiments looked at the differ-

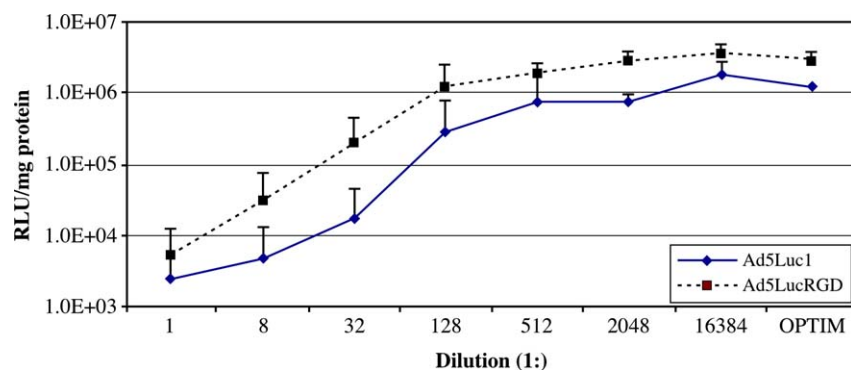


Fig. 3. Triple immunization with Ad5, and neutralization of Ad5RGD and Ad5. Mice ($n = 5$) were challenged sequentially three times by Ad5Luc1. Collected sera on day 27 were employed to block Ad5Luc1 and Ad5LucRGD before infection of SKOV3 ip1 cells. A luciferase assay was carried out 48 h post-infection. Data represent the mean of quadruplicates (t -test, $P = 0.0043$).

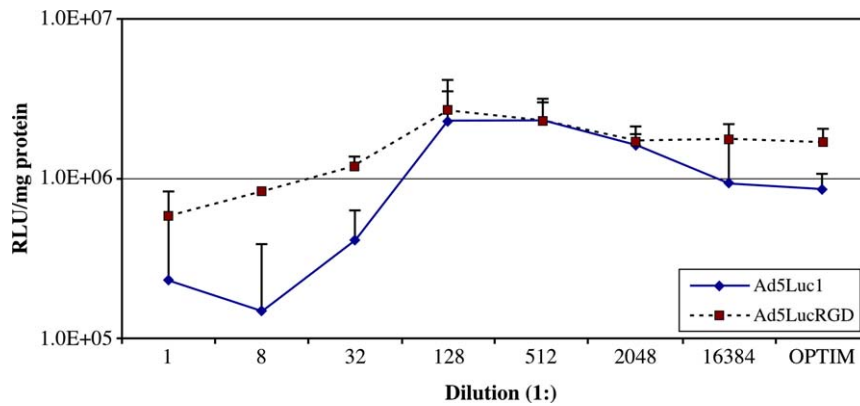


Fig. 4. One immunization with Ad5RGD, and neutralization of Ad5RGD and Ad5. Mice ($n = 5$) were challenged once by Ad5LucRGD. Collected sera on day 27 were employed to block Ad5Luc1 and Ad5LucRGD before infection of SKOV3 ip1 cells. A luciferase assay was carried out 48 h post-infection. Data represent the mean of quadruplicates (t -test, $P = 0.06$).

ential in transfection with NAb exposure for both RGD modified and unmodified viruses from that achieved without NAb exposure. It is the difference in this differential and not the actual baseline differential that is, we believe, important.

In this report, all mice had a low but measurable titer of NAb (Fig. 1). Although an adenovirus-associated acute cellular immune response has previously been observed in mice [28], any preexistent anti-Ad humoral immunity has not been documented. Our previous data showed a lower level of NAb existed in all experimental mice compared to human beings [3] living in a high exposure environment [29]. The preexistent NAb had a higher neutralizing activity to AdLuc1 than to Ad5LucRGD. Thus, Ad5LucRGD is seen as a more effective gene transfer system compared to Ad5Luc1 in the presence of preexisting anti-ad Ad immunity. The explanation for the modest inhibitory effects of the serum from naive mince on viral infection is unclear, but may be related to nonspecific anti-viral properties present in serum. It is important to note that in regard to

the data used to generate this figure, Opti-MEM indicates no serum and was used to normalize the infection volume.

As noted, an enhancement in gene transduction efficiency by Ad5LucRGD was accomplished in human ovarian cancer cell lines and in primary human ovarian cancer cells [20,21]. The results of our studies comparing the titers of anti-Ad5Luc1 and anti-Ad5LucRGD NAb of mice after single and triple immunization by Ad5Luc1 and Ad5LucRGD, respectively (Figs. 2 and 3), showed that the humoral immune response triggered by Ad5Luc1 produced a stronger NAb to Ad5Luc1 than to Ad5LucRGD, which retained a neutralization preference for AdLuc1 over AdRGDLuc1, as preexistent NAb. In the presence of anti-AdLuc1 NAb, the Ad5LucRGD-mediated gene transduction was more efficient than Ad5Luc1.

The higher in vivo gene delivery efficacy of Ad5LucRGD (Figs. 4 and 5) is correspondent with previous in vitro data [20,21]. This achievement was attributed to the modified tropism, which allows the virus to alternatively binding to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin receptors.

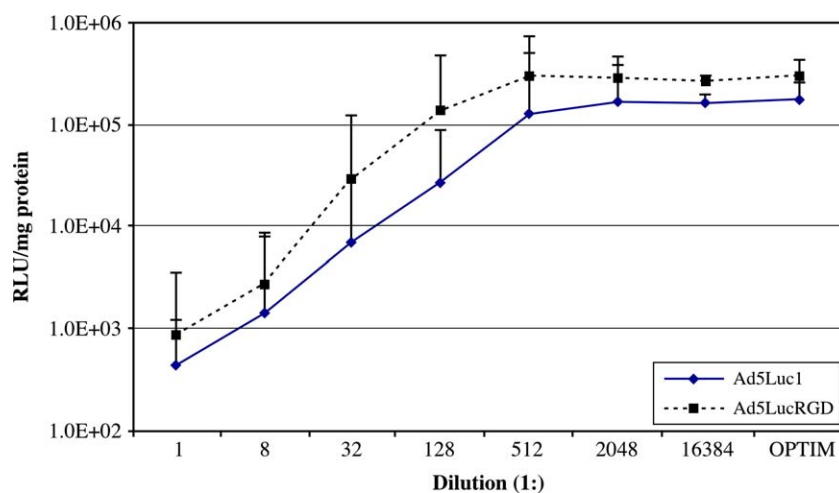


Fig. 5. Triple immunization with Ad5RGD, and neutralization of Ad5RGD and Ad5. Mice ($n = 5$) were challenged sequentially three times by Ad5LucRGD. Collected sera on day 27 were employed to block Ad5Luc1 and Ad5LucRGD before infection of SKOV3 ip1 cells. A luciferase assay was carried out 48 h post-infection. Data represent the mean of quadruplicates (t -test, $P = 0.0069$).

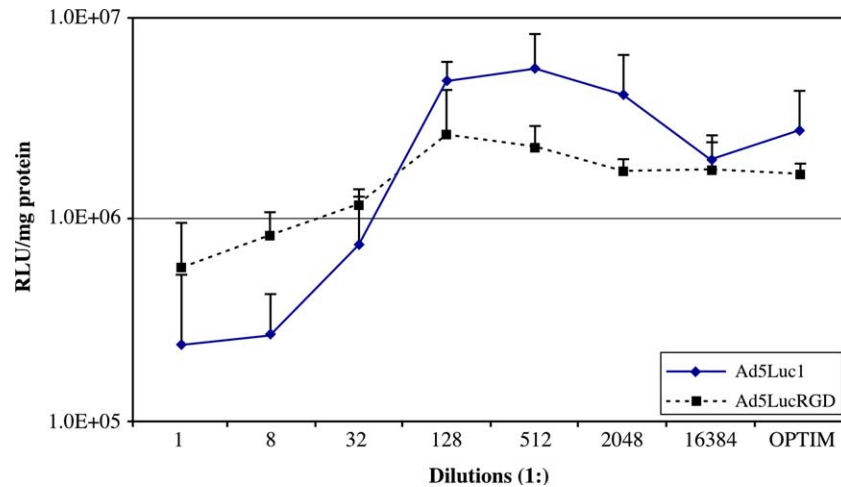


Fig. 6. One immunization with Ad5RGD and Ad5, respectively, neutralization of Ad5RGD and Ad5, respectively. Mice ($n = 5$) were challenged once by Ad5Luc1 (same data from Fig. 2) and Ad5LucRGD (same data from Fig. 4), respectively. Anti-Ad5Luc1 and anti-Ad5LucRGD sera from day 27 were employed to block Ad5Luc1 and Ad5LucRGD, respectively, before infection of SKOV3. ip1 cells. A luciferase assay was carried out 48 h post-infection. Data represent the mean of quadruplicates (t -test, $P = 0.031$).

NAb are composed of different molecular subpopulations that react to different epitopes on the adenovirus including the hexon, penton base, and fiber [2,30–33]. Among the above-cited capsid proteins, hexon and fiber are antigenic epitopes for serotype specificity [34]. Because the binding of the fiber knob domain to the cellular receptor is a key event for Ad infection [35,36], anti-fiber antibodies can inhibit adenovirus infection [30–32]. In our report, both vectors shared the same backbone and reporter gene, the sole difference between the amino acid sequences residing on the HI loop of the fiber knob [37]. Ad5Luc1 has an unmodified knob and is a CAR-dependent vector system. On SKOV3.ip1, a low CAR and high integrin cell line [20], the obviously inhibitory gene delivery by Ad5Luc1 may be secondary to the anti-fiber subpopulation of the anti-

AdLuc1 pool. On the contrary, AdRGDLuc1 has an RGD-modified fiber knob. Serotype specificity of an adenovirus is determined by γ -determinant of the fiber knob and ϵ -determinant of hexon [34]. The incorporation of an Arg–Gly–Asp (RGD)-containing peptide in the HI loop of the fiber knob domain not only renders an alternative infection pathway to the vector but also most probably changes the antigenic epitopes that result in an alteration of antigenicity. Therefore, the anti-AdLuc1 NAb had a limited inhibitory effect on Ad5LucRGD.

However, the titer of anti-Ad5LucRGD NAb from one challenge of Ad5LucRGD is higher than the titer of anti-AdLuc1 from one challenge of Ad5Luc1 (Fig. 6). But there is no significant difference between the two vectors with three challenges (Fig. 7). These results suggest that

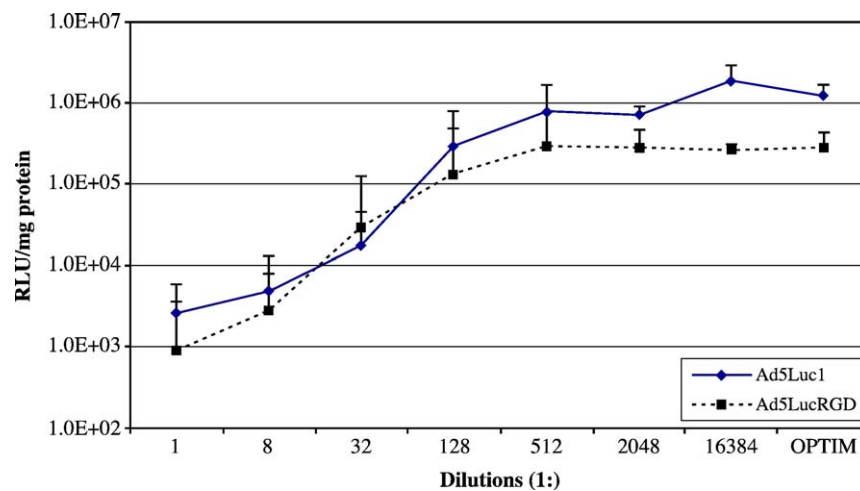


Fig. 7. Triple immunization with Ad5RGD and Ad5, respectively, neutralization of Ad5RGD and Ad5, respectively. Mice ($n = 5$) were challenged three times by Ad5Luc1 (same data from Fig. 3) and Ad5LucRGD (same data from Fig. 5), respectively. Anti-Ad5Luc1 and anti-Ad5LucRGD sera from day 27 were employed to block Ad5Luc1 and Ad5LucRGD, respectively, before infection of SKOV3. ip1 cells. A luciferase assay was carried out 48 h post-infection. Data represent the mean of quadruplicates (t -test, $P = 0.2135$).

Ad5LucRGD may possess a stronger antigenicity than AdLuc1. Since the fiber knob of Ad plays an important role in the determination of serotype specificity and immunogenicity [34,37], it follows that incorporation of an Arg–Gly–Asp (RGD)-containing peptide in the HI loop of the fiber knob domain might change the antigenic epitopes, resulting in an alteration of antigenicity. The potential advantages for such antigenic alteration in Ad5LucRGD are that Ad5LucRGD may evade the recognition and neutralization by preexisting NAb, which mainly neutralize unmodified Ad. On the other hand, we feel that the antigenicity of Ad5LucRGD and resulting anti-AdRGDLuc1 NAb formed in response to exposure are an important safety feature that may help protect patients from side effects.

An intriguing finding was the higher gene transfer with Ad5LucRGD (in comparison to Ad5Luc1), even when mice were immunized with Ad5LucRGD. Further studies are needed to clarify the reasons for this, but this could be related to reduced capacity of the immune system in forming NAb against fiber conformations not found in nature. Moreover, a contributing factor could be tighter binding of the RGD-modified fiber to integrins, which could be advantageous in an environment with Nabs. Although the interpretation of NAb data comes indirectly from speculation based on luciferase readings and does not represent actual titer of NAb, they functionally show the neutralizing capability of immunized sera. NAb may directly affect gene transfer and subsequent protein production and are relevant from the translational therapeutic standpoint. In conclusion, RGD-modified viruses may be useful gene transfer agents when preexisting anti-Ad5 neutralizing antibodies are present.

Acknowledgments

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