Development of stealth transgenes for gene therapy : evaluation of cis-acting inhibitors of antigen presentation

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Chapter 2

Creation of immune ‘stealth’ genes for gene therapy through fusion with the Gly-Ala repeat of EBNA-1

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RESEARCH ARTICLE

Creation of immune ‘stealth’ genes for gene therapy through fusion with the Gly-Ala repeat of EBNA-1

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A major obstacle in gene-therapy protocols is T-cell-mediated destruction of transgene-expressing cells. Therefore new approaches are needed to prevent rapid clearance of transduced cells. We exploited the Gly-Ala repeat (GAr) domain of the Epstein–Barr virus nuclear antigen-1, since the GAr prevents cytotoxic T-lymphocyte-epitope generation. Here we show that three different enzymes (viz. the E. coli LacZ gene encoded b-galactosidase, firefly luciferase, and HSV1 thymidine kinase) fused with the GAr retained their function. Moreover, linking GAr with b-galactosidase successfully prevented recognition of GAr-LacZ-expressing cells by b-galactosidase-specific CTL. Nonetheless, vaccination with a GAr-LacZ adenovirus or with an allogeneic cell line expressing GAr-LacZ resulted in the induction of b-gal-specific CTL. This demonstrates that the GAr domain does not inhibit crosspresentation of antigens, but only affects breakdown of endogenously synthesized proteins. These data demonstrate how the GAr domain can be exploited to create immuno‘stealth’ genes by hiding transgene products from CTL-mediated immune attack.

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Introduction

The transfer of genes holds promise as a therapeutic approach for the treatment of a wide variety of diseases. One of the limitations imposed onto gene therapy is the immune response directed against vector and/or trans-gene product.[1–4] While beneficial for the development of recombinant vaccines against infectious agents[5,6] and tumor cells,[7,8] it significantly impedes the development of those gene-therapy approaches where persistent expression of the transgenes encoding neoantigens is required. Long-term humoral and cellular immunity against several viral-vector systems prevails in a large part of the population, or may be induced upon the first vector administration. This may frustrate (re)administration of the vector and lead to elimination of the transduced cells. Developments in the viral and nonviral technology greatly improved the efficiency of the gene-transfer vectors. Retroviral, lentiviral, adenoviral, and adeno-associated viral vectors are available from which all virus-derived protein-coding genes have been re-moved.[9] This eliminated, albeit not completely,[10] the problem associated with the cellular-immune response against the vector-derived antigens.

However, a cellular immune response against neoantigens encoded by the transgene may still be induced. This problem has been most prominently described following adenovirus-mediated transfer of the E.coli-derived LacZ gene, encoding b-galactosidase. Strong immune responses against β-galactosidase have been observed in rodents following adenovirus-mediated gene transfer into liver, muscle, lung, and brain, leading to local inflammation, destruction of the transduced cells, and loss of transgene expression.[4,11–13] Antigen-specific major histocompatibility complex (MHC) class-I restricted cytotoxic T lymphocytes (CTL) are the prime suspects responsible for target cell destruction.[13–16] This requires prior activation of $CD4^+$ T cells.[1,16–23] Also in primates a CTL response directed against the transgene product has been shown to occur.[24,25] In a clinical trial aiming at inducing a graft versus leukemia response, eight of 24 treated patients developed a specific cytotoxic $CD8^+$ T-cell-mediated immune response against the cells genetically engineered to express the Herpes Simplex Virus 1 (HSV1) thymidine-kinase (TK) gene. This led to the selective elimination of the modified cells.[25]

Previous attempts to reduce the T-cell responses against the neoantigens during gene therapy focused on blocking the MHC class I-and class II-restricted T-cell responses, or the prevention of costimulation of T cells.[14,18–20,26–28] However, these approaches were either not fully effective or resulted in a general immunosuppression. The ideal strategy would selectively prevent the presentation by MHC class I of the transgene-derived peptides.

Under normal circumstances, the $CD8^+$ CTL response plays an important role in the control of virus infections, generating effector cells that kill infected cells upon recognition of viral peptides presented by MHC class I molecules. Given the important role of CTL in the control of virus-spread, it is no surprise that viruses have developed numerous strategies that frustrate and abrogate antigen presentation.[29,30] One such example comes from the Epstein–Barr Virus (EBV) nuclear-antigen 1 (EBNA-1). EBNA-1 is expressed in latent EBV-infected cells. Here it is indispensable for the virus as it is required for the maintenance of the viral episomes. Although EBNA-1-specific CTL have been described in infectious mononucleosis patients and healthy carriers, they cannot recognize EBV-infected cells.[31–36] The failure to recognize endogenously expressed EBNA-1 has been attributed to the glycine-alanine repeat (GAr) domain in the EBNA-1 sequence that protects EBNA-1 from proteasomal degradation and subsequent presentation in the context of MHC class I.[37,38] This successful immune-evasion strategy points to the unique opportunity to hide cells expressing transgenes from CTL-mediated target-cell destruction by incorporation of the GAr sequence into the transgene.

Here, we show that inclusion of the full EBNA-1-derived GAr domain does not inhibit enzyme function. A recombinant adenovirus expressing the GAr-containing LacZ gene as its transgene was able to deliver a functional enzyme after infection in mice invivo, resulting in the induction of a strong β-galactosidase-specific CTL response in the recipient mice. However, these murine antigen-specific CTL did not recognize cells expressing the GAr-transgene fusion, demonstrating that the EBNA-1-derived GAr can be exploited to create 'stealth' transgenes by hiding the transgene-expressing cells from CTL-mediated immune-attack.

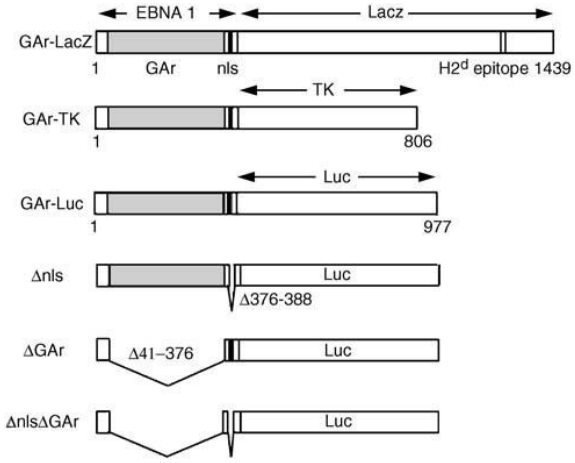

Figure1

Schematic outline of the chimaeric Gly-Ala repeat constructs. Indicated are the Gly-Ala repeat unit (GAr; aminoacids 90–328) and the nuclear localization signal (nls, aa 378–386). The open reading frames of *E.coli* β-galactosidase, HSV1-TK, and firefly luciferase start at aa 427. In the C-terminal part of LacZ, the H-2^d CTL epitope is indicated (aa 1303–1311). Also indicated are the deletions of the GAr region and the nls that were made in luciferase.

Results

GAr does not inhibit protein function

To study whether the GAr domain can be exploited to enhance the persistence of gene expression by minimizing CTL-mediated recognition of transgene-expressing cells, we first tested whether enzymes retained their function if fused with the full-length GAr domain. To this end, we constructed plasmids encoding GAr-β-galactosidase (pGAr-LacZ), GAr-HSV-thymidine kinase (pGAr-TK), and GAr-luciferase (pLXRN-GAr-Luc; Figure 1). Transfection experiments revealed that the functional properties of the enzymes were not affected as evidenced from β-galactosidase activity in Hep2 cells (Figure 2a), [^{3}H]thymidine incorporation and ganciclovir sensitivity in Rat2 cells (Figure 2b), and luciferase activity in 911 cells (Figure 2c), respectively. Thus, these data indicate that the GAr does not inhibit the function of proteins harboring the full-length GAr. A slight (three-fold) reduction in luciferase activity can be attributed to the presence of the GAr in the transcription unit, and a mere two-fold reduction to the nls. However, functionally the vectors are equivalent to the unmodified predecessors.

Delivery of GAr-LacZ by a recombinant adenovirus

As recombinant viruses most efficiently facilitate gene transfer, we generated a recombinant adenovirus (rAd5) with the GAr-LacZ gene (rAd5-GAr-LacZ) to test the stability of the repeats in the adenovirus backbone. Whereas manipulation of plasmids with

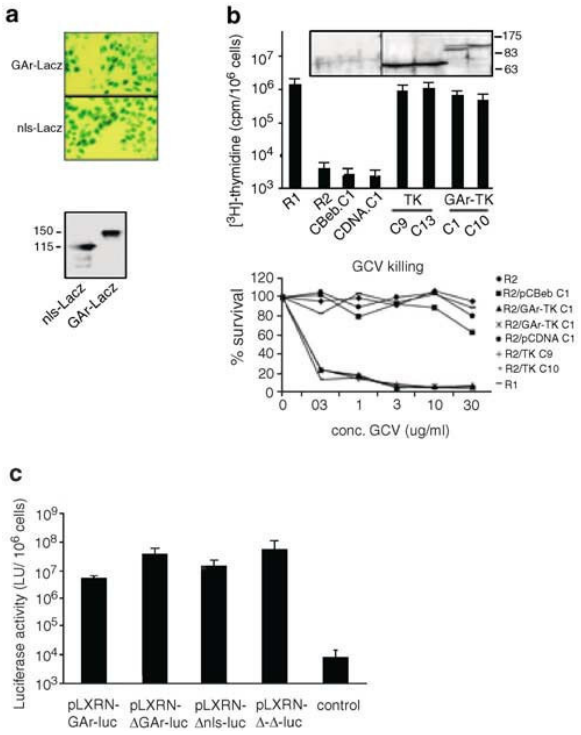

Figure 2

GAr fusion enzymes retain their activity. (a) Activity of GAr-β-galactosidase. Hep2 cells were infected with rAd5-nls-LacZ or rAd5-GAr-LacZ (4 PFU/cell). At 48h postinfection, the cells were fixed and stained for β-galactosidase activity. In addition, H_{1299} cells grown in 10-cm dishes were infected with rAd5-nlsLacZ or rAd5-GArLacZ with 10 PFU/cell. After 48h, protein extracts were made and size-fractionated by SDS-PAGE, transferred to nitrocellulose membranes and probed with an anti-LacZ mouse monoclonal antibody (Roche, Basel, Switzerland). (b) Activity of GAr-TK. The TK-deficient cell line Rat2 (R2, TK^-) was used to generate stable cell lines containing the various plasmids. CBeb.C1 and CDNA.C1 are G418-resistant Rat2 cells obtained after transfection of the empty pCBeb and pCDNA3.1 plasmids, respectively. GArTK.C1 and C10 are independent G418-resistant clones of Rat2 cells stably expressing the pGAr-TK plasmid. TK.C9 and C13 are two independent G418-resistant Rat2 clones derived by transfection of the plasmid pCDNA-TK. Rat1 (R1) is the TK^+ parental cell line from which Rat2 had been derived. [^{3}H] thymidine incorporation (± s.d.) is represented per 10^6 cells. The inset depicts a Western analysis of the Rat2 clones with a polyclonal goat anti-HSV-TK antibody. The faster-migrating band in clone GAr-TK.C1 may result from splicing within the TK coding region.[59] The same cell lines were analysed for their ganciclovir sensitivity by growing these cells for 48h in the presence of varying concentrations of ganciclovir. Cell viability was determined with the WST-I colorimetric assay. (c) Activity of GAr-luciferase. Cultures of 911 cells were transfected with pCBeb (as a negative control), pLXRN-GAr-luc and the GAr- and nls-deletion derivatives. Cells were lysed 18h post-transfection and the luciferase activity was measured in the lysates. The mean of three experiments is shown, expressed as light units/10^6 cells ± 1 s.d.

the full-length GAr proved difficult as the repeats lead to frequent internal deletions in the GAr domain (DJMvdW and MO, unpublished observations), rAd5 vectors carrying the GAr-LacZ fusion gene could be generated and be propagated with titers similar to those of vectors lacking the GAr domain. Western analysis of infected cells detected β-galactosidase at the expected molecular weights of 150 kDa for GAr-LacZ and 115 kDa for nls-LacZ (Figure 2a). This confirms that the entire GAr-β-galactosidase fusion protein was synthesized. Infection of

Hep2 cells with rAd5-nls-LacZ as well as rAd5-GAr-LacZ with 4 plaque-forming units (PFU)/cell yielded β-galactosidase activity in approximately 50% of the cells 2 days after infection (Figure 2a). Similarly, intracellular staining revealed that the amount of β-galactosidase present after infection of Hep2 cells with rAd5-GAr-LacZ with 10 PFU/cell was similar to the amount observed after infection with rAd5-nls-LacZ (data not shown). Thus, rAds harboring the GAr repeats can be stably propagated and used to deliver efficiently functional GAr fusion genes to cells.

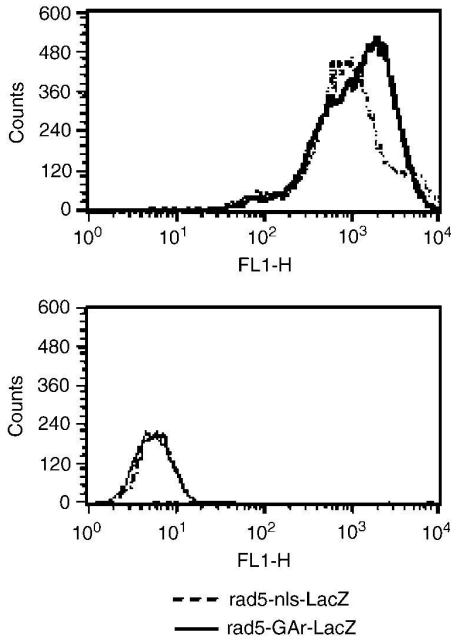

Figure 3
Delivery by rAd leads to the expression of the entire GAr-LacZ with the same efficiency as delivery of nls-LacZ. To determine the efficiency of gene delivery, MEC were infected with 10 PFU/cell of rAd5-GAr-LacZ and rAd5-nls-LacZ, respectively. At 2 days postinfection, the β-galactosidase activities were determined by intracellular FACS staining with the fluoreporter LacZ flowcytometry kit (Molecular Probes Inc., Eugene, OR, USA). The dotted line represents the signal in rAd5-nls-LacZ-infected cells, the solid line depicts the signal in rAd5-GAr-LacZ-infected cells. In the lower panel, the negative controls are shown for both cell populations.

GAr inhibits recognition of LacZ-expressing cells by CTL

As recognition of transgene expressing cells by CTL is an important limitation for prolonged transgene expression invivo, we investigated whether target cells infected with rAd5-GAr-LacZ could present the H-2L^d-restricted CTLepitope Lac876–884 (TPHPARIGL)[39] to LacZ-specific CTL. Therefore, BALB/c mouse embryo cells (MEC) were infected with either rAd5-nls-LacZ or rAd5-GAr-LacZ. After 2 days, when both infected MEC populations expressed similar transgene levels as determined by intracellular FACS staining (Figure 3), as well as by determination of β-galactosidase activity (data not shown),

the cells were used as stimulators for LacZspecific CTL. LacZ-expressing MEC but not GAr-LacZ-expressing MEC were efficiently recognized by LacZ-specific CTL as determined by an interferon-γ production assay (Figure 4). The GAr-LacZ-expressing and LacZ-expressing MEC did not differ in MHC expression and the GAr-LacZ-expressing MEC were efficiently recognized by LacZ-specific CTL after loading with $Lac_{876–884}$ peptides (data not shown). These findings indicate that, despite similar transgene expression, the GAr inhibits the generation of CTL epitopes derived from LacZ.

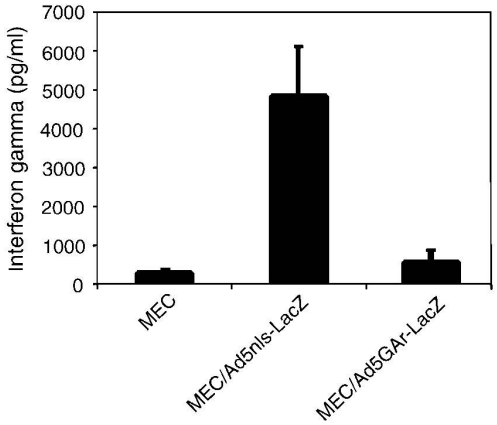

Figure 4
GAr prevents the presentation of the LacZ-encoded CTL epitope TPHPARIGL to LacZ-specific CTL. Xc9 MEC were mock-infected, infected with 5 PFU/cell rAd5-nls-LacZ or rAd5-GAr-LacZ. After 2 days, at the time when 10 000 LacZ-specific CTL were added to 15 000 infected cells (E:T ratio 1:1.5), the LacZ expression of MEC infected with rAd5-nls-LacZ or rAd5-GAr-LacZ was similar to LacZ expression as determined by intracellular FACS with a fluoreporter LacZ flowcytometry kit (Figure3). After 24h CTL activation was determined by measuring the IFN-γ produced by LacZ-specific CTL in the supernatant. Xc9 MEC infected with rAd5-nls-LacZ but not with rAd5-GAr-LacZ are efficiently recognized by β-galactosidase-specific CTL.

Induction of β-galactosidase specific CTL

The above studies show that GAr-LacZ-expressing target cells are poorly recognized by β-galactosidase-specific CTL. To study whether the GAr fused with β-galactosidase would also inhibit CTL induction *in vivo*, we immunized mice with rAd5-GAr-LacZ as well as with rAd5-nls-LacZ viruses (10^8 PFU/mouse), as controls. At 21 days postinjection, splenocytes were harvested and analyzed for the presence of β-galactosidase-specific CTL. Mice vaccinated with either rAd5-nls-LacZ and rAd5-GAr-LacZ viruses mounted a strong β-galactosidase-specific CTL response (Figure 5). Together, these data indicate that the GAr does inhibit CTL recognition but not CTL induction.

Since presentation of β-galactosidase-derived CTL epitopes by GAr-LacZ-expressing target cells is strongly inhibited by the action of the GAr, it is conceivable that CTL directed against GAr-containing proteins are primed via an indirect pathway, which is not hampered

by the activity of the GAr. To study the mechanism responsible for priming of CTL directed against GAr-containing antigens, we set out to vaccinate BALB/c mice (H-2^d) with GAr-LacZ-expressing C57BL/6 (H-2^b) cells unable to present the LacZ epitope to β-galactosidase-specific CTL directly. MEC from C57BL/6 mice were infected with 1 PFU/cell rAd5-GAr-LacZ. After 2 days, the cells were thoroughly washed, irradiated,[40] and used for vaccination of the completely allogeneic BALB/c mice (H-2^d). As the allogeneic H-2^b MHC expressed by the immunizing MEC cannot prime H-2^d-restricted host CTLs directly, generation of β-galactosidase-specific CTL requires crosspriming, that is, the uptake and H-2^d-restricted representation of antigen by host antigen-presenting cells (APC). Vaccination of BALB/c mice with the completely allogeneic vaccine resulted in the induction of a β-galactosidase-specific CTL response, indicating that GAr-LacZ crossprimes CTL (Figure 6). To exclude the possibility that BALB/c anti-C57BL/6 allospecific CTL induced by immunization of BALB/c mice with C57BL/6 cells crossreacted on the β-galactosidase epitope, we vaccinated BALB/c mice with uninfected C57BL/6 cells. These mice, however, did not generate β-galactosidase-specific immunity (Figure 6).

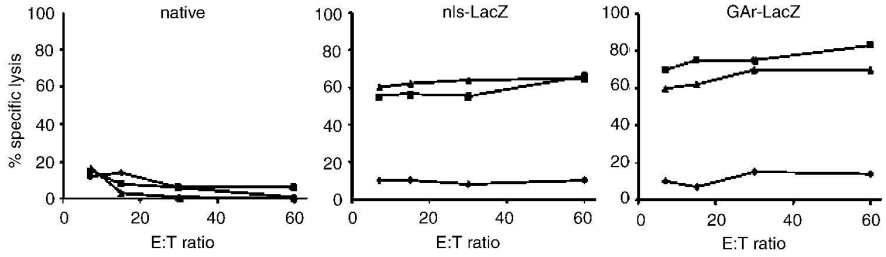

Figure 5

Vaccination with rAd5-GAr-LacZ leads to induction of a strong β-galactosidase-specific CTL response. BALB/c mice were naive, were immunized with 10^8 PFU rAd5-nls-LacZ or with 10^8 PFU rAd5-GAr-LacZ by intraperitoneal administration. After 3 weeks, β-galactosidase-specific CTL activity was analysed in a cytotoxicity assay on BALB/c MEC (H-2^d; control, ♦), LacZ-peptide-loaded BALB/c MEC (▲), and LacZ-transfected BALB/c MEC (■). The rAd5-GAr-LacZ-immunized animals have mounted a β-galactosidase-specific CTL response similar to rAd5-nls-LacZ vaccinated animals.

We were unable to isolate detectable amounts of rAd5-GAr-LacZ virus from the C57BL/6 MEC 2 days postinfection (<0.01 PFU/cell) by washing or freeze– thawing the vaccine cells. Hence the maximum amount of free viruses administered with the vaccine (<400 PFU) is well below the minimal amount of viruses required to induce an immune response (>10^6 PFU/ mouse; data not shown). Thus the induction of the immune response cannot be attributed to carry-over of free rAd5-GAr-LacZ vector viruses with the vaccine. Taken together, these data indicate that GAr-β-galactosidase crossprimes CTL and explain

why LacZ-specific CTL induction is not blocked, whereas recognition of GAr-LacZ-expressing cells is inhibited by the action of the GAr.

Prolonged LacZ expression in vivo by delivery of GAr-containing LacZ

We have demonstrated that while the GAr domain protects antigens from destruction by antigen-specific CTL *in vitro*, it does not inhibit the induction of antigen-specific CTL *in vivo*. To assess whether insertion of the GAr will result in prolonged transgene expression *in vivo*, we injected either rAd5-GAr-LacZ or rAd5-nls-LacZ into the gastrocnemius muscle of two groups of BALB/c mice (H-2^d). At days 8 and 19 postinjection, the muscles were analyzed for β-galactosidase activity as a read-out for transgene expression. At day 19, β-galactosidase-positive cells are present in the muscle transduced with rAd5-GAr-LacZ. In contrast, the β-galactosidase-positive cells were eradicated in the rAd5-nls-LacZ-injected muscle, demonstrating that the GAr prolongs transgene expression *in vivo*(Table 1). Similar results were obtained after injection of rAd5-GAr-LacZ in the gastrocnemius muscle of the right leg and rAd5-nls-LacZ left leg of the same mouse (Table 2), excluding a bias due to difference in immunity of the GAr-LacZ and nls-lacZ proteins. Thus, the GAr-β-galactosidase persists longer than the nls-LacZ-derived control protein.

Table 1
Intramuscular β-galactosidase expression at various times after vector administration

Day	rAd5-GAr-LacZ	rAd5-nls-LacZ	rAd5-nls-LacZ (nu/nu)
8	4(4)	4(4)	1(1)
19	4(4)	1(4)	1(1)

The vectors rAd5-GAr-LacZ or rAd5-nls-LacZ were injected (10^8 PFU/injection) into the left gastrocnemius muscle of two groups of BALB/c mice (H-2^d) and as controls into BALB/c *nu/nu* mice. At days 8 and 19, the muscles were taken out and sections were stained for β-galactosidase activity, as a read-out for transgene expression. No β-galactosidase expression was discernable in the contralateral muscles. The values represent the number of mice showing β-galactosidase activity at the site of injection and the total number of mice tested (in parenthesis).

Table 2
Intra-animal comparison of intramuscular β-galactosidase expression after adenovirus-mediated transfer of nls-LacZ and GAr-LacZ

Day	rAd5-GAr-LacZ	rAd5-nls-LacZ	rAd5-nls-LacZ (nu/nu)
8	3(3)	2(3)	1(1)
19	3(3)	0(3)	1(1)

The vectors rAd5-GAr-LacZ and rAd5-nls-LacZ were injected (10^8 PFU/injection) into the left and right gastrocnemius muscle of BALB/c mice (H-2^d), respectively. BALB/c *nu/nu* mice served as controls. At days 8 and 19, the muscles were taken out and sections were stained for β-galactosidase activity. The values represent the number of mice showing β-galactosidase activity at the site of injection and, the total number of mice tested (in parentheses).

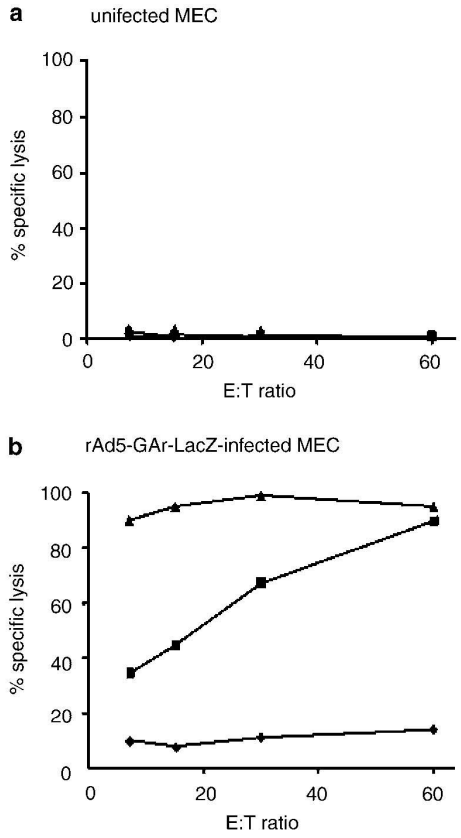

Figure 6
Vaccination of BALB/c mice with rAd5-GAr-LacZ-infected allogeneic cells induces LacZ-specific CTL. BALB/c mice were immunized with uninfected (a) or 4×10^4 rAd5-GAr-LacZ-infected MEC derived from a C57BL/6 mouse (b). After 3 weeks, LacZ-specific CTL activity was analyzed. The β-galactosidase-specific CTL activity was analysed in a cytotoxicity assay on BALB/c MEC ($H\text{-}2^d$; control, ♦), LacZ-peptide-loaded BALB/c MEC (▲), and LacZ-transfected BALB/c MEC cells (■). Mice vaccinated with infected C57BL/6 MEC, but not immunized with uninfected MEC, have mounted a strong β-galactosidase-specific CTL response, indicating that the GAr does not inhibit crosspriming of CTL.

Discussion

In this study, we evaluated the use the EBNA1-derived GAr element to prevent presentation of transgene-derived *neo*antigens. We provide three examples where fusion of the GAr domain did not impede the function of three popular reporter proteins. We also show that the GAr element does not affect induction of transgene-directed CTL activity upon adenovirus-mediated transfer and expression of a GAr-LacZ fusion gene. However, recognition of transgene-expressing cells by antigen-specific CTL is abolished sufficiently to prevent cytolysis by β-galactosidase-specific CTL.

The mode of action employed by the GAr domain to inhibit antigen processing is not fully understood. Studies by Levitskaya *et al*[37] showed that the GAr domain of EBNA-1 acts as a cis-inhibitory activity of MHC class I-restricted presentation. The GAr domain inhibited the ubiquitin/proteasome-dependent proteolysis pathway by interference with protein folding or otherwise inhibiting the capacity of the target protein to bind components of the ubiquitin-dependent proteasome pathway.[38] Recent studies demonstrate that small synthetic GAr-derived peptides do not inhibit polyubiquitination, but rather inhibit stable association of the protein with proteasomes.[41] However, the effect of these peptides on antigen presentation was not determined.[42,43] Subsequent studies have shown that cotranslational ubiquitination of EBNA-1 can override the GAr-mediated inhibition of proteasomal degradation and restore the endogenous processing and presentation of MHC class I-restricted CTL epitopes.[44] This suggests that the GAr domain not only prevents proteasomal degradation but also ubiquitination of GAr domain-containing proteins. Alternatively, experiments with p53 GAr fusion proteins suggest that the GAr domain acts on events between ubiquitination and proteasomal degradation.[45] The observation that the GAr does not inhibit priming of CTL is important and consistent with reports describing the presence of EBNA-1-specific CTL in EBV-seropositive individuals.[31–36] As antigen presentation by professional APC, most likely DC, is crucial to the initiation of virus-specific CTL responses, the presence of EBNA-1-specific CTL in EBV-positive donors suggests that antigen processing for MHC class I by specialized APC is not hampered by the GAr. In case EBV-specific CTL are induced through the direct route following EBV infection of DC, these observations would indicate that antigen presentation in DC differs intrinsically from presentation by 'nonprofessional' APC. Alternatively, EBV-specific CTL could have been primed in an indirect manner (crosspriming), as this is likely representing the dominant way by which CTL responses are induced *in vivo*.[40,46,47]

In case EBV-specific CTL are indeed primed in an indirect manner following uptake of viral antigens derived from EBV-infected cells, the presence of EBNA-1-specific CTL points to the possibility that the GAr does not affect the processing of exogenously acquired EBNA-1-antigens.[34–36] The GAr domain does not inhibit CTL priming via the indirect pathway, as vaccination with completely allogeneic tumor cells expressing GAr-LacZ resulted in a strong LacZ-specific CTL response. As these completely allogeneic tumor cells lack the proper MHC class I restriction element, generation of Lac-Z-specific CTL must involve uptake and H-2^{d}-restricted re-pre-sentation of antigen by host APC. Although not the subject of this study, it is tempting to speculate that the (GAr-containing) antigens taken up by DC are initially processed in endosomal/lysosomal compartments resulting in the liberation of the GAr from the antigenic CTL epitope. In this way, the processing of the CTL epitope is separated from the inhibitory influence of the GAr on proteasomal antigen degradation and

would explain the observation that CTL priming proceeds in an uninhibited fashion, while recognition of GAr-LacZ-expressing target cells is severely inhibited.

The immune response against *neo*antigens encoded by the vector or by the transgene represents a major limitation for the successful clinical application of gene therapy for the treatment of chronic diseases where long-term transgene expression is desired. Currently, vector systems are being developed from which all vector-derived protein-coding regions have been removed. Nonetheless, the immune response against the transgene product itself is not circumvented by these approaches, limiting the persistence of transduced cells. As CTL-mediated immune attack is the most important effector mechanism responsible for the destruction of transgene-expressing cells, we sought to determine whether transgene-expressing cells could be rescued by the insertion of the GAr in a transgene of interest. Indeed, prolonged transgene expression was detected in mice treated with rAd5-GAr-LacZ, consistent with the prominent role of the cellular immunity to transgene-encoded products on the persistence of transgene expression.[3] Nonetheless, we did not achieve persistent transgene expression *in vivo*, which is likely to be the result of the use of the first-generation E1-deleted adenoviral vectors. These vectors are notorious for their immunogenicity due to the leaky expression of viral genes residing in the vector.[3,48]

The GAr provides a powerful and specific tool to inhibit the presentation of transgene-derived antigens to $CD8^+$ CTL. Provided that the GAr is also functional in human cells, it may be exploited in gene-therapy applications involving expression of new antigenic proteins, for example, in hereditary protein deficiencies, in enzyme/prodrug or 'suicide' strategies that employ bacterial or viral enzymes, and in hiding bacterial transcription-regulating proteins, that is, those used in the tetracycline-regulated gene-expression systems. Since the GAr specifically targets the proteasome pathway and therefore the destruction of transgene-expressing cells via $CD8^+$ CTL, it is questionable if the system works as well for secreted and proteolytically activated proteins like blood-clotting factors FVIII and FIX. Although this study did not look into this, it is unlikely that the GAr system will be effective in such application. Whereas the longevity of the cells making the secreted proteins may increased, fragments of the secreted proteins may still be processed and presented despite the presence of the GAr.

The GAr approach may eliminate the risks that are associated with other immune suppressive approaches that have been described. Many of these are not antigen specific and aim at a more general immunosuppression, which enhances the risk of opportunistic infections that cannot be counteracted by the immune system. In contrast, the GAr provides an antigen-specific approach to temper CTL-mediated immune destruction, as is also evident from the effectiveness by which EBV exploits the GAr to prevent CTL-mediated destruction of EBNA1-expressing B cells in humans.

Materials and methods

Construction of the GAr fusion constructs

The LacZ gene carrying the EBNA-1 Gly-Ala repeats was constructed by inserting the LacZ gene into plasmid pCMV-EBNA (Invitrogen, Groningen, The Netherlands) (Figure 1). Firstly, plasmid pCBeb was created from plasmid pCMV-EBNA by converting the BstXI site to a *Bgl*II site by insertion of a *Bgl*II adapter that was created by annealing the synthetic oligonucleotides 5'-TACGAGATCTGAAG-3'and 5'-AGATCTCGTACTTC-3' (the *Bgl*II site is underlined). Secondly, a 3072 bp *Bam*HI fragment of the retroviral vector pBag,[49] which carries the LacZ-coding region except the first two codons, is inserted into the *Bgl*II site of pCBeb to generate pGAr-LacZ.

Similarly, the plasmids pGAr-TK and pGAr-Luc were created. For the first, a PCR was performed on the wtHSV1 (strain17) DNA to obtain the TK gene. Primers TK-for (5'-CAGGATCCTGACCATGGCTTCGTACCCCT GCCATC-3') and TK-rev (5'-GTGGATCCTGATCAGTTAGCCTCCCCCATCTCCCG-3') containing extensions with *Bam*HI restriction sites (underlined) were used and subsequently the TK gene was cloned into the pCBeb plasmid. For the latter, another PCR was performed on pCMVluc, a plasmid containing the luciferase gene. Primers Cl.luc-for (5'-GCAGATCTCCATGGAAGACGCCAAAAACAT-3') and Cl.luc-rev (5'-GCAGATCTC GAGCTAGCTCAATTTGGACTTTCCGCC-3') containing *Bgl*II sites (underlined) were used and the fragment cloned into pCBeb. The modified GAr-luc gene was inserted into vector pLXRN, in which the Mo-MuLV LTR promoter drives expression of the luc gene. The nls and GAr region were deleted by PCR mutagenesis using the QuikChange Site-directed Mutagenesis Kit (Stratagene Europe, Amsterdam, The Netherlands) according to the manufacturer 's protocols. In all plasmids, the presence of the sequences coding for the GAr was verified by restriction analyses and DNA sequencing, as these repeats negatively affected the replication of the plasmid DNA and, as a result, had a tendency to be lost during propagation. The control plasmid pCDNA-TK contains the unmodified HSV-TK cDNA.

Adenoviral vector construction

The adenoviral vector pAd-GAr-LacZ was constructed following the procedures described.[50] The CMV-EBNA-LacZ cassette was excised from pGAr-LacZ as a 5125 bp *Bam*HI–*Hin*dIII fragment and inserted into plasmid pShuttle-CMV,[50] digested with *Bgl*II and *Hin*dIII, to create pSEbLacZ. The adenoviral vector plasmid pAd5-GAr-LacZ was made by homologous recombination between pSEbLacZ and pAdEasy1[50] in *E.coli* BJ5183. To generate recombinant viruses, *Pac*I-digested pAd5-GAr-LacZ was transfected into 911 cells.[51] In all experiments, the vector rAd5-nls-LacZ served as a control. This vector contains the LacZ codons fused with the SV40 large T-derived nuclear localization signal (nls). Amplification, propagation, and screening for replication-competent adenovirus were performed as described.[52,53]

Plaque assays were performed essentially as described.[51] Briefly, adenovirus stocks were serially diluted in 1 ml DMEM/2% HS and added to near-confluent 911 cells in six-well plates. After 2 h of incubation at 37°C/5% CO2, the medium was replaced by agar-containing culture medium. All batches were checked for integrity of the transgene by PCR and Southern analysis.

Western analyses

H_{1299} cells were infected with rAd5-nlsLacZ or rAd5GArLacZ with 10 PFU/cell. After 48 h, protein extracts were made and size-fractionated by SDS-PAGE, transferred to Immobilon-P nitrocellulose membranes, and probed with an anti-LacZ mouse monoclonal antibody (Roche, Basel, Switzerland). After incubation with a peroxidase-conjugated goat-anti-mouse secondary antibody, the protein was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). Similarly, Rat2 clones were analyzed for the presence of HSV-TK. Protein extracts were made and 50 mg was size-fractionated by SDS-PAGE, transferred to Immobilon-P nitrocellulose membranes, and probed with a rabbit anti-HSV-TK antiserum (1/250 diluted), kindly provided by Dr M Janicot (Aventis-Gencell, Vitry-sur-Seine, France) and visualized with a peroxidase-conjugated goat-anti-rabbit secondary antibody.

Enzymatic assays

Luciferase and β-galactosidase activity assays were performed as described.[54,55] The [^{3}H]thymidine incorporation assays and the ganciclovir sensitivity assays to detect HSV-TK activity were performed in Rat2 cells as described.[56] Cell viability was assessed with the WST-1 colorimetric assay (Roche diagnostics, Almere, The Netherlands) according to the manufacturer 's description.

Generation and analysis of CTL bulk cultures

BALB/c mice were vaccinated by intraperitoneal injection as described in the legends to the figures. After 3 weeks, β-galactosidase-specific CTL were generated as follows: 5×10^{6} spleen cells per well were cocultured for 6 days with 10% irradiated BALB/c-derived stimulator cells expressing the LacZ gene (P13.1 cells)[57] in 24-wells plates. Next, effector cells were harvested and dead cells were removed by density centrifugation on Lympholyte M (Cedarlane, Hornby, Canada). These cells were used in a cell-mediated lymphocyte cytotoxicity assay as described previously.[58]

LacZ expression in vivo

At day 0, 12 Balb/c mice received an injection into the gastrocnemius muscle of one leg with 1×10^{8} PFU rAd5-nls-LacZ. Another group of 12 Balb/c mice received i.m. in one leg with 1×10^{8} PFU rAd5-GAr-LacZ. Additionally, three nude mice were injected i.m. with 1×10^{8} PFU rAd5-nls-LacZ in one leg and 1×10^{8} PFU rAd5-GAr-LacZ in the other. At days 8 and 19, four animals of each group and a nude mouse were killed and the gastrocnemius muscles were isolated from both legs. As negative control a naive mouse was killed, whereas the nude mouse served as positive control.

Alternatively, six Balb/c mice and two Balb/c$^{nu/nu}$ nude mice were injected i.m. with 1×10^{8} PFU rAd5-nls-LacZ in one leg and 1×10^{8} PFU rAd5-GAr-LacZ in the other. These muscles were collected at days 8 and 19 (three Balb/c and one nude). A naive mouse served as negative control. In both protocols, the muscles were flash frozen in TissueTek using liquid nitrogen. Sections (10 mm) that were cut were either directly stained or stored at −80°C until further use.

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