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Transfectant Influenza A Viruses Are Effective Recombinant Immunogens in the Treatment of Experimental Cancer

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Abstract

Using reverse genetics methods, we constructed three different transfectant influenza A viruses encoding an L^d-restricted, nine amino-acid-long fragment, corresponding to amino-acid residues 876–884, of *β*-galactosidase (*β*-gal). Sequences encoding this epitope were nested within the hemagglutinin (HA) or neuraminidase (NA) open reading frames. Alternatively, an Independent *β*-gal mini-gene, preceded by an endoplasmic reticulum insertion signal sequence, was placed in a bicistronic arrangement in the NA RNA segment of the virus. All three transfectants mediated the presentation of the epitope to a *β*-gal-specific CTL clone. Furthermore, each of the three transfectant viruses expressing the *β*-gal fragment elicited specific cytolytic responses *in vivo*. Most importantly, these H1N1 transfectants mediated the regression of established murine pulmonary metastases. Tumor regression in mice was also achieved in the presence of preexisting immunity against an H3N2 influenza A virus serotype. Nononcogenic and nonintegrating, transfectant influenza A viruses are attractive candidates for development as antitumor vaccines.

INTRODUCTION

There is strong evidence suggesting that the stimulation of a potent and specific immune response against tumor cells will result in tumor clearance. Data from mice and human suggest that T lymphocytes are an important element of that response, and the antigens recognized by T cells have, in some cases, been identified (Greenberg, 1991; Kawakami and Rosenberg, 1997). While cancer cells may express proteins designated tumor-associated antigens (TAA), growing cancers generally do not elicit TAA-specific immune responses that are capable of mediating their rejection. Attempts to increase the poor immunogenicity of tumor cells have included physical modification of the tumor cells (including γ-irradiation), the inoculation of mixtures of tumor cells and pathogens (viruses, bacteria, and bacterial extracts), and more recently, gene-modification of the tumor cells with a variety of immunomodulatory molecules.

In contrast to tumor cells, most viruses are powerful inducers of cellular immune responses. An entirely new and potentially more effective strategy for eliciting immune responses against TAA involves the insertion of the recently cloned genes encoding TAA into recombinant viruses (reviewed in (Restifo, 1996)). These recombinant viruses are then used as therapeutic vaccines to induce immune responses against tumor cells. A number of recombinant expression vectors have been shown to be useful in the prevention and, in some cases, the treatment of tumors in experimental animals including poxviruses [vaccinia (Moss, 1996; Bronte *et al.*, 1997), MVA (Carroll *et al.*, 1997), fowlpox (Wang *et al.*, 1995), and canary pox (Kawakita *et*

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al., 1997)], adenoviruses (Randrianarison-Jewtoukoff and Perricaudet, 1995; Chen *et al.,* 1996), polioviruses (Andino *et al.,* 1994; Ansardi *et al.,* 1994), and nonviral vectors including plasmid DNA administered by injection and by "gene gun" (Irvine *et al.,* 1996).

Despite the potential application of these vectors to the treatment of patients with cancer, the reasons for the continued exploration of new vectors are threefold. First, although important properties of anticancer vectors can be deduced (e.g., safety, efficacy, ease of engineering, genetic stability and the lack of oncogenicity, integration, and immunosuppression), therapeutic efficacy in the clinic has yet to be demonstrated with any of the vectors now available. Second, preexisting immune responses in cancer patients to some of the vectors listed above can hinder their function as recombinant immunogens. For example, neutralizing antibodies can be present in the patients as a result of previous infection with a wild-type form of the vector (e.g., adenovirus) or as a result of prior immunization (e.g., vaccinia virus or poliovirus). Third, the availability of a number of vectors makes novel prime/boosting protocols possible (Rodrigues *et al.,* 1994; Murata *et al.,* 1996). Prime-boost combinations employing non-cross-reactive vectors can be much more effective than combinations that employ repeated doses of a single vector, perhaps due to the presence of vector-specific antibodies following the first immunization.

In this report, we explore the potential of transfectant influenza A virus vectors for use in the immunotherapy of cancer. The genetic manipulation of influenza viruses is now possible, and we have developed reverse genetics techniques that allow us to generate stable recombinant (transfectant) viruses expressing foreign epitopes and/or polypeptides (Palese *et al.,* 1996). Influenza A viruses are strong inducers of cellular immune responses. Strikingly, mice immunized with an influenza virus vector and boosted with a vaccinia virus vector expressing the same antigen are able to generate specific cytolytic $CD8^+$ T lymphocytes against the expressed antigen that represent approximately 1% of the total splenic $CD8^+$ cells (Murata *et al*., 1996). Influenza A viruses are nonintegrating, nononcogenic viruses. There are a number of nontransmissible attenuated strains of influenza viruses available that could be used in humans (Chanock and Murphy, 1980; Maassab *et al.,* 1988). Since influenza viruses change their antigenic determinants very quickly, one could also choose among different viral strains to avoid the presence of preexisting immunity against the virus in patients.

We have tested the efficacy of transfectant influenza viruses expressing a TAA to clear tumors in a murine cancer model. The tumor used in our studies is the experimental murine tumor CT26, which has been transfected with the *lacZ* gene [which encodes *β*-gal, used in this system as a model TAA (Wang *et al*., 1995)]. We engineered transfectant influenza viruses expressing a CTL epitope from *β*-gal, then determined the ability of these viruses to induce a therapeutic cellular immune response in mice bearing experimental tumors.

RESULTS

Rescue of transfectant influenza A viruses encoding a T cell epitope from *β*-galactosidase

Recombinant viruses encoding $CD8^+$ T cell antigenic determinants derived from cancer cells might mediate the regression of tumors *in vivo* (Irvine *et al*., 1995; McCabe *et al.,* 1995; Restifo, 1996). We thus constructed transfectant influenza A viruses encoding TPHPARIGL, which corresponds to amino acids 876–884 in the intact *β*-gal protein. This determinant is presented by the MHC class I L^d-molecule on the surface of our experimental tumor, CT26.CL25 (Gavin *et al.,* 1993; Wang *et al*., 1995).

Three different influenza A virus transfectants were generated (Fig. 1). The first transfectant virus, called MINIGAL, contains a minigene in a bicistronic arrangement within the NA-specific viral RNA segment. mRNA derived from this segment uses a mammalian internal

ribosomal entry site (IRES) placed downstream of the minigene and upstream of the NA gene to achieve translation of the NA protein. The minigene is translated following the usual cap-dependent initiation of translation in eukaryotic cells. In this transfectant virus, the minigene was engineered to encode the E3/19K leader sequence at the N terminus of the *β*-gal epitope TPH-PARIGL. The ER-insertion signal sequence has been found, in some cases, to greatly.augment the immunogenicity of the encoded epitope (Restifo *et al.*, 1995). The control virus for this construct, designated BIP-NA virus, employs the same IRES element upstream of the NA open reading frame but does not contain the *β*-gal epitope minigene (Garcia-Sastre *et al*., 1994). The second virus, NAGAL, encodes the amino-acid sequence TPHPARIGL inserted in the stalk region of the NA protein. The control for this construct is the MNA transfectant virus, which contains the irrelevant peptide SYVPSAEQI inserted into the NA stalk. This sequence is derived from the CS protein of *Plasmodium yoelii* (Rodrigues *et al.*, 1994). The third virus, called BHAGAL, encodes the *β*-gal epitope inserted into the antigenic site B of the HA protein. The control for this virus, designated ELDKWAS, contains the gp41 HIV-derived sequence ELDKWAS inserted into the same domain of the HA (Muster *et al.*, 1995).

Transfectant viruses MINIGAL, NAGAL, and BHAGAL were rescued following RNP transfections into helper influenza virus-infected cells. Sequence analysis of the rescued viruses confirmed the presence of the foreign *β*-gal-derived sequences. Viral titers obtained in MDBK cells for the transfectant viruses expressing the *β*-gal-epitope were comparable to the control transfectant viruses BIP-NA, MNA and ELDKWAS and approximately 1 log lower than wild-type influenza A/WSN/33 virus.

Transfectant influenza virus-infected cells specifically present the *β*-gal-epitope to CD8$^+$ T cells

To ascertain if the *β*-gal-epitope expressed by the transfectant influenza viruses could be processed and presented in the context of MHC class I molecules, CT26.WT tumor cells were infected with the different transfectant influenza A viruses encoding the *β*-gal epitope TPHPARIGL or the control viruses. Infected cells were then co-incubated for 24 h with a CD8$^+$ T lymphocyte clone specific for this epitope. Supernatants were then assayed for GM-CSF, and the results are shown in Fig. 2. Cells that were infected with MINIGAL, NAGAL, and BHAGAL viruses elicited specific release of GM-CSF. Neither control transfectant virus- or wild-type virus-infected cells were recognized by the *β*-gal-specific CTLs. Thus the transfectant influenza A viruses were found to mediate the expression- of the L^d-restricted *β*-gal epitope in forms that could be processed and presented at the surface of infected cells.

Transfectant influenza A viruses elicit a *β*-gal specific cytolytic response in mice

Cytolytic responses mediated by CD8$^+$ T lymphocytes specific for TAA play an important role in the regression of established tumors in both mice and humans (Greenberg, 1991; Rao *et al.*, 1996; Rosenberg, 1997). To evaluate the function of transfectant influenza A viruses in the priming of *β*-gal-specific cytotoxtc responses *in vivo*, we immunized mice with the panel of influenza A viruses. Three weeks later, splenocytes from immunized mice were cultured in the presence of the L^d-restricted *β*-gal$_{876–884}$ peptide for 6 days and subsequently tested in a microcytotoxicity assay. Cultured splenocytes from mice immunized with the three transfectant influenza A viruses expressing the *β*-gal epitope (MINIGAL, NAGAL, and BHAGAL viruses) were capable of specific recognition of CT26.CL25 cells or of CT26.WT cells pulsed with synthetic peptide (Fig. 3). No specific recognition was elicited by wild-type virus or by the control transfectant viruses.

Treatment of 3-day-old established tumors by vaccination with transfectant influenza A viruses

Specific cytolytic responses were elicited in mice by the transfectant influenza A viruses expressing the β-gal$_{876–884}$ peptide. To evaluate whether these responses had any impact on the growth of tumor cells, we immunized mice bearing CT26.CL25 tumors established for 3 days with our panel of recombinant immunogens. These immunogens were active, in the absence of the T cell growth factor IL-2, previously shown to increase the efficacy of recombinant poxvirus-based vaccines (Bronte *et al.*, 1995). As shown in Fig. 4, treatment of mice with MINIGAL, NAGAL, or BHAGAL viruses resulted in a statistically significant reduction of the number of lung metastases measured at 12 days (P_2 values for Experiments 1 and 2, respectively, were 0.005 and 0.004 for BIP-NA *vs* MINIGAL; 0.045 and 0.007 for MNA *vs* NAGAL; 0.039 and 0.005 for ELDKWAS *vs* BHAGAL). In some instances, treated mice did not show any macroscopic evidence of lung tumors by Day 12. The results indicate that transfectant influenza A viruses expressing a single tumor antigen determinant were able to induce a therapeutic antitumor response in mice.

Antitumor responses elicited by transfectant H1N1 influenza viruses in mice prelmmunized with an H3N2 influenza virus

Preexisting immunity against a viral vector might prevent the induction of cellular immune responses against its encoded antigens. Thus we investigated whether a transfectant influenza virus expressing a tumor-associated antigen is able to elicit similarly efficient antitumor responses in naive and in influenza-virus-immune animals. Groups of mice were first intranasally infected with H1N1 or H3N2 influenza viruses. We used the attenuated NA/B-NS transfectant virus and a mouse-adapted influenza A/Beijing/32/92 virus as the H1N1 and H3N2 immunogens, respectively. The NA/B-NS virus is antigenically identical to influenza A/WSN/33 virus, but it is attenuated due to the presence of specific mutations in the promoter of one of its genes. NA/B-NS virus (10^3 PFU) induces a protective immune response against influenza virus without killing the infected mice (Muster *et al*., 1991). Three weeks postinfection, all mice immunized with the NA/B-NS virus had HI antibody titers in sera against influenza A/WSN/33 virus but not against influenza A/Beijing/32/92 virus (data not shown). Reciprocally, all mice immunized with influenza A/Beijing/32/92 virus had HI antibody titers in sera against influenza A/Beijing/32/92 virus but not against influenza A/WSN/33 virus. This was expected since the hemagglutinin proteins of both virus are antigenically different. H1N1-immune mice bearing CT26.CL25 tumors did not respond to treatment with a transfectant H1N1 influenza virus (NAGAL) expressing the β-gal$_{876–884}$ peptide. However, despite the presence of cross-reactive cellular epitopes in the internal proteins of H1N1 and H3N2 viruses, the ability of NAGAL virus to mediate regression of established CT26.CL25 tumors was not impaired in H3N2-immune mice (Fig. 5).

DISCUSSION

We have shown in this communication that treatment of tumor-bearing mice with transfectant influenza A viruses expressing a model TAA can result in a significant reduction of the number of lung metastases measured 12 days later. The experimental murine cancer used in these studies relies on the use of the cell line CT26.CL25. This cell line is derived from the carcinoma cell line CT26, which was transduced to express β-gal (Wang *et al*., 1995). In this murine cancer model, the tumorigenic properties of the cell line CT26.CL25 remain unchanged upon expression of the model TAA β-gal. This resembles the situation in most human tumors, which express TAA but are not able to induce an immune response against their TAA. However, in contrast to the mouse model that was used in our studies, human cancers are established for longer periods of time in the patients. In addition, most of the identified human TAA are derived from melanoma cells, and they are also expressed in normal melanocytes. Issues of tolerance

are likely to bedevil attempts at inducing responses to self antigens. An interesting consequence of the breaking of tolerance in these conditions may be vitiligo, a patchy and permanent loss of pigmentation in the skin, which has been observed in some of our patients with melanoma who respond positively to melanoma. Importantly, we have not observed any ocular or aural consequences to these apparent "self" reactivities (Rosenberg and White, 1996). Although the CT26.CL25 tumor model does not address all problems that one will encounter when treating a human cancer, it is still very promising that influenza viruses expressing a single antigenic determinant can mediate the regression of established tumors in mice.

We have engineered three influenza virus vectors expressing the same β-gal epitope in different contexts. Among these three transfectant viruses, BHAGAL virus, which expresses the β-gal epitope in the context of the HA gene, is expected to express higher levels of the epitope than the other two viruses, MIIMIGAL and NAGAL, which express the β-gal epitope in the context of the NA gene. This is because there are about four to five times more copies of HA than there are NA on the surface of an influenza A virus (Lamb and Krug, 1996). On the other hand, the MIIMIGAL virus may deliver the epitope to MHC class I molecules more efficiently due to the use of a leader sequence in front of the epitope (Restifo *et al*., 1995). Finally, one might also expect differences in the efficiency of processing of the β-gal epitope according to the different flanking amino acid sequences that are present in the three viral vectors. However, all three viruses were able to induce a therapeutic immune response against tumors expressing β-gal in mice.

In clinical cancer trials at the National Cancer Institute and elsewhere, the recombinant viral vectors that are currently in use include E1-deleted adenoviruses and recombinant poxviruses that encode selected human melanoma associated antigens (MAA) (Rosenberg, 1997). It was shown previously that adenovirus- and poxvirus-based vectors were also able to induce tumor inhibition in experimental murine cancer models (Wang *et al*., 1995; Chen *et al*., 1996; Bronte *et al*., 1997; Carroll *et al*., 1997). Clinical work has recently shown that most cancer patients have high circulating levels of neutralizing titers to the adenovirus vectors that we are using. While humans generally do not have preexisting immunity to the avian poxviruses, the vast majority of patients have immunity to vaccinia viruses, since they have generally received the virus as children (a result of the world-wide effort by the World-Health Organization to eradicate smallpox). Indeed, many patients will also have neutralizing antibodies to many different influenza A virus strains. The presence of neutralizing antibodies against the viral vectors in patients will more likely result in a lower level or viral replication and of MHC class I presentation, thus decreasing the induction of tumor specific $CD8^+$ T cells. However, humans are susceptible to repeated bouts of influenza because influenza viruses can almost endlessly change the antigenic characteristics of their viral coats. Tumor immunotherapists could then choose a viral coat to which cancer patients were not immune, for example, because the patient was not yet born at the time the chosen viral coat type was pandemic. The latter case, for example, could involve the use of influenza virus vectors expressing selected TAA (preferentially in a gene segment encoding an internal viral protein) and bearing a viral coat from 1934 (such as influenza A/PR/8/34 virus) to treat a population born after that date. This strategy is supported by the experiments shown in Fig. 5. Thus although the antigenically distinct H3N2 and H1N1 influenza viruses share conserved $CD4^+$ and $CD8^+$ epitopes, an H1N1 transfectant virus is able to efficiently induce antitumor responses in mice which were previously immunized with an H3N2 influenza virus.

Another possible advantage of the use of influenza virus vectors to express TAA is their antigenic simplicity. Influenza A virus encodes only 10 proteins, as compared to the 185 open reading frames of vaccinia virus, some of which are known to encode immunosuppressive proteins such as soluble, secreted homologues of cytokine receptors. The proportion of the expressed desired antigen among other viral antigens is higher for influenza virus than for

poxvirus-based vectors. Future experiments are in progress to carefully compare the potency of different vectors expressing TAA, including vaccinia and influenza virus-based vectors.

The capacity of influenza A viruses to infect dendritic cells and to express their genes at high levels is also an important one. Dendritic cells are potent activators of T lymphocyte-dependent immune responses. They have a high density of both class I and class II MHC molecules on their surfaces together with costimulatory molecules like B7-1/CD80 and B7-2/CD86, as well as other T cell activating ligands including ICAM-1/CD54. Dendritic cells infected with influenza viruses expressing TAA *ex vivo*, then reinfused, could be used to activate antitumor T cells -*in vivo* (Steinman, 1996). In fact, the elicitation of potent antitumor immunity described in this manuscript might be mediated by the infection of dendritic cells *in vivo*, as shown in the case for recombinant vaccinia virus-based immunogens (Bronte *et al*., 1997). Greenberg and colleagues have recently used an *ex vivo* approach in which they employ recombinant vaccinia virus infected cells to generate antityrosinase reactivities (Yee *et al*., 1996). Likewise, we have raised cytotoxic T lymphocytes specific for MART-1/MelanA using poxvirus-infected dendritic cells (Kim *et al*., 1997). However, vaccinia viruses are about 20-fold more antigenically complex and thus may be more likely to elicit irrelevant reactivities than an influenza A virus vector.

An interesting and important consideration in the development of recombinant anticancer vaccines has to do with boosting studies. We and others have found that repeated administrations of the same vector to boost cellular immune responses are usually not successful (Murata *et al*., 1996; Irvine, 1997). Thus the first administration of the vector results in the induction of neutralizing antibodies against the vector that hamper its ability to subsequently reinfect the same patient. This could be circumvented by combined immunizations with two different vectors sharing the same TAA. A very promising protocol involves the use of an influenza virus vector to prime an immune response against the expressed TAA, followed by a poxvirus vector expressing the same TAA for boosting. We have previously shown that this protocol of immunization is extremely efficient in mice for the induction of powerful specific CTL responses against foreign malarial antigens that are expressed by the vectors (Murata *et al*., 1996).

Safety is one major concern in the use of influenza virus vectors in humans. Temperature-dependent host range phenotypes exist for a variety of viruses, including influenza A viruses (Chanock and Murphy, 1980; Maassab *et al*., 1988; Crowe *et al*., 1995; Snyder *et al*., 1990). The use of nontransmissible, attenuated cold-adapted influenza virus vectors could provide a means to safely administer the vector to humans. These cold-adapted strains were obtained by propagation of the virus at progressively lower temperatures, resulting in the selection and accumulation of mutations responsible for both cold-adaptation and attenuation. Alternatively, the administration of transfectant influenza viruses by routes different from the respiratory route could also provide a safe way to use these vectors in humans. Influenza A viruses productively infect the respiratory epithelium but cause neither viral shedding nor disease when administered by nonrespiratory routes, e.g., intravenously, intraperitoneally, intramuscularly, or subcutaneously. However, these routes of administration are equally effective as the intranasal route in eliciting a cellular immune response against expressed antigens by the influenza virus vectors in mice (Murata *et al.*, 1996). In fact, the mouse immunizations described in this communication were performed intravenously or intraperitoneally, and they resulted in both an induction of CTLs against the model TAA and in tumor inhibition. Finally, it is also possible to construct by reverse genetics techniques stable attenuating mutations in the chimeric viral gene expressing the TAA. In fact, insertion of foreign antigens into the influenza virus genome usually results in the attenuation of the viral vector. Issues of safety will nevertheless require further study.

In conclusion, transfectant influenza viruses expressing a single antigenic determinant encoded by a minigene, or embedded within a structural protein, can elicit powerful cellular immune responses and have therapeutic efficacy against tumors established for 3 days in mice. This is the first example of a negative-strand RNA virus vector that is able to induce antitumor immune responses. To explore the use of these new vectors in human cells *in vitro*, we recently engineered transfectant influenza viruses expressing the melanoma TAA MART-1, as well as two immunogenic fragments of the gp100 molecule. To create viruses that can be used in the clinic, we are exploring the use of strains of influenza A virus to which there are little or no preexisting humoral immune responses. Such viruses could be most useful in the development of prime-boost combinations with other recombinant vectors. Our challenge is to identify viruses that remain safe, immunogenic, and nontransmissible when given to humans with cancer.

MATERIALS AND METHODS

Animals, viruses and cell lines

Six- to eight-week-old female BALB/c (H-2^d) mice were obtained from Frederick Cancer Research Center (Frederick, MD). CT26 is an N-nitroso-N-methylurethrane-induced BALB/c (H-2^d) undifferentiated colon carcinoma. The cloning of this tumor cell line to produce CT26.WT and the subsequent transduction with *lacZ* and subcloning to generate CT26.CL25, which stably expresses *β*-gal, has been described previously (Wang *et al*., 1995). These cell lines were maintained in RPMI 1640,10% heat inactivated FCS (Biofluids, Rockville, MD), 0.03% L-glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 50 μg/ml gentamicin sulfate (NIH Media Center). In addition, 400 or 800 μg/ml G418 (GIBCO, Grand Island, NY) was added to the maintenance media of the CT26.CL25 cells. Madin-Darby bovine kidney (MDBK) cells were used for growing wild-type, influenza A/WSN/33 (H1N1) virus and for rescuing and growing H1N1 transfectant influenza viruses. Attenuated transfectant NA/B-NS influenza virus (H1N1) was previously described (Muster *et al*., 1991). MDBK cells were maintained in reinforced minimal essential medium containing 10% heat inactivated FCS (GIBCO). Influenza A/Beijing/32/92 virus (H3N2) was adapted to mice by passaging seven times in the lungs of BALB/c mice. Mouse adapted influenza A/Beijing/32/92 virus was grown in 10-day-old embryonated chicken eggs.

Construction and characterization of transfectant influenza A viruses

The construction of the transfectant influenza viruses BIP-NA, MNA, and ELDKWAS, which were used in control experiments, has been described (Rodrigues *et al*., 1994; Garcia-Sastre *et al*., 1994; Muster *et al*., 1994). Transfectant viruses which express the L^d-restricted *β*-gal epitope TPHPARIGL were obtained by RNP-transfection as previously described (Garcia-Sastre and Palese, 1993). These viruses contain one RNA segment that is derived from genetically engineered plasmid cDNA encoding the neuraminidase (NA) or hemagglutinin (HA) genes of influenza A/WSN/33 virus. One virus, called MINIGAL, encodes the amino-acid sequence MRYMILGLLALAAVCSAATPHPARIGL from a minicistron followed by a mammalian internal ribosomal entry site (IRES) element just upstream of the NA open reading frame. Amino-acid residues in front of the *β*-gal epitope TPHPARIGL are derived from the leader peptide of the E3/19K protein (Restifo *et al*., 1995). A control virus, BIP-NA, contains the same IRES sequences upstream of the NA open reading frame but lacks the *β*-gal minicistron (Garcia-Sastre *et al*., 1994). The second transfectant virus, NAGAL, encodes the amino-acid sequence TPHPARIGL inserted in the stalk region of the NA protein. The third transfectant influenza virus, BHAGAL, encodes the same *β*-gal epitope inserted into the antigenic site B of the viral HA protein. MNA and ELDKWAS viruses, which contain irrelevant epitope insertions in the same context as NAGAL and BHAGAL viruses, respectively, were used as controls. Transfectant viruses were plaque purified three times in

MDBK cells, and their identities were subsequently confirmed by RT-PCR and sequencing of gene regions containing the engineered foreign sequences (Garcia-Sastre *et al*., 1994).

Peptides

The synthetic peptide TPHPARIGL was synthesized by Peptide Technologies (Washington, DC) to a purity of greater than 99% as assessed by HPLC and amino-acid analysis. This peptide represents the naturally processed H-2 L^d-restricted epitope spanning amino acids 876–884 of *β*-gal (Gavin *et al*., 1993; Wang *et al*., 1995).

^{51} Chromium release assays

Six-hour ^{51}Cr release assays were performed as previously described (Restifo *et al*., 1993). Briefly, 2 [times 10^6 target cells were incubated with 200 mCi $Na^{51}Cr0_4$(^{51}Cr) for 90 min. Peptide-pulsed CT26.WT cells were incubated with 1/ng/ml of synthetic peptide during labeling. Target cells were then mixed with effector cells for 6 h at the effector to target (E:T) ratios indicated. The amount of ^{51}Cr released was determined by γ-counting, and the percentage of specific lysis was calculated from triplicate samples as follows: [(experimental cpm-spontaneous cpm)/(maximal cpm - spontaneous cpm)] ×100.

In vitro stimulation of *β*gal-specific cytotoxic T cells

CT26.WT cells, 10^5/well in 96-well U-bottom plates (Costar) were incubated in complete medium (RPMI, 0.1% BSA, and 30 mM HEPES at pH 6.8) and infected with the influenza viruses shown at an m.o.i. of 20 for 3 h. CTL_X were then added at an E:T of 1. Note that CTL_X is a clone specific for a peptide with the sequence TPHPARIGL corresponding to the naturally processed epitope from *β*-gal (spanning amino acids 876–884) and presented by the H-2 L^d molecule. After 24 h of coincubation, supernatants were harvested and assayed for GM-CSF using a commercially available ELISA kit (R&D, Minneapolis, MN). GM-CSF levels were used as a measure of $CD8^+$ T cell reactivity.

In vivo experiments

Active treatment studies involved BALB/c mice inoculated intravenously with 5×10^5 CT26.CL25 cells. Three days later, mice were randomized and then inoculated intravenously or intraperitoneally with,10^6 PFU of the indicated transfectant or wild-type influenza virus. Twelve days after tumor injection, mice were ear tagged, randomized again, and sacrificed. Lung metastases were enumerated in a blinded fashion by an investigator with no knowledge of the experimental groups. Preimmunizations of mice were done as follows: mice were inoculated intranasally with 10^3 PFU of influenza NA/B-NS virus or of mouse-adapted influenza A/Beijing/32/92 virus. Three weeks after immunization, sera were collected, and the presence of antibodies against influenza A/WSN/33 and A/Beijing/32/92 viruses was determined using a hemagglutination inhibition (HI) assay.

Statistical analysis

Data concerning to the number of lung metastases do not follow a normal distribution (since all lungs that contain >250 metastases were deemed too numerous to count) and thus were analyzed using the nonparametric two-tailed Kruskal-Wallis test. All statistical values expressed are P_2 (two-tailed *P)* values.

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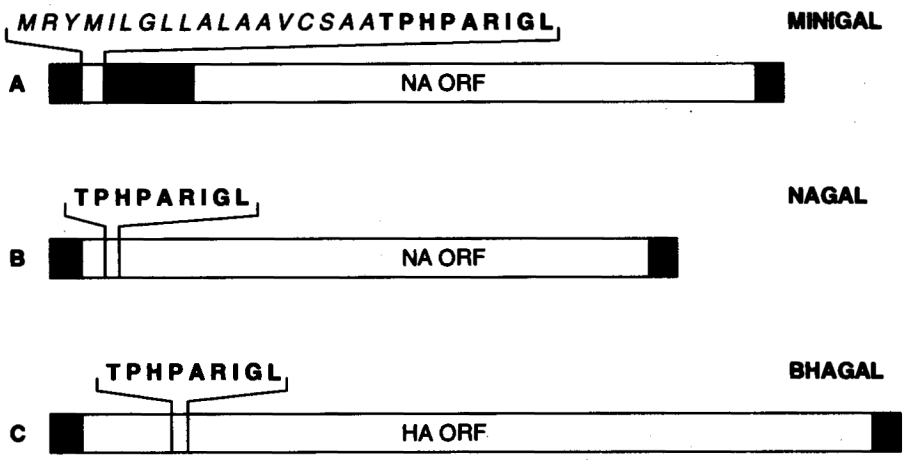

FIG. 1.

Schematic representation of the recombinant genes of the transfectant influenza viruses expressing the $CD8^+$ T-cell β-gal epitope TPHPARIGL (A) MINIGAL recombinant gene. The β-gal epitope is expressed downstream of a leader peptide (characters in italics) as an independent polypeptide from a bicistronic NA gene. Expression of the viral NA protein in this gene is achieved via internal initiation of translation mediated by an IRES element derived from the BiP mRNA (Garcia-Sastre *et al.*, 1994). (B) NAGAL. The β-gal epitope is expressed as part of the amino acid sequence of the NA protein. (C) BHAGAL The β-gal epitope is expressed as part of the amino acid sequence of the HA protein. The NA and HA open reading frames (ORF) are indicated. Black boxes represent noncoding regions in the represented genes.

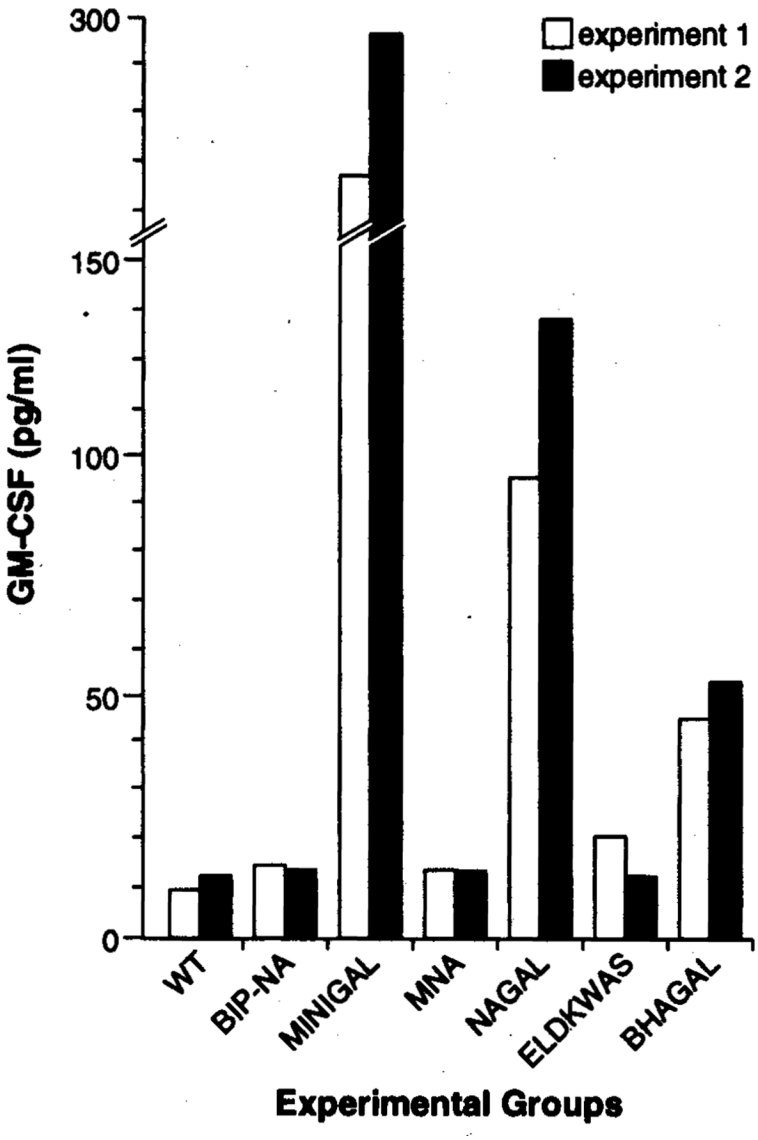

FIG. 2.

Specific recognition of transfectant influenza A viruses-infected cells by a *β*-gal-specific CTL clone. Five × 10^5 CT26.WT cells/well (24-well plate) were incubated in RPM1, 0.1% BSA, and 30 mM HEPES at pH 6.8, and they were infected with the influenza viruses shown at an m.o.i. of 5 for 3 h, then washed with CM and plated with CTL_X, which are specific for the *β*-gal epitope TPHPARIGL, at an E:T ratio of 1. After 24 h of coincubation, cell Supernatants were harvested and assayed for GM-CSF. Results from two independent experiments are represented. WT, influenza A/WSN/33 wild-type virus.

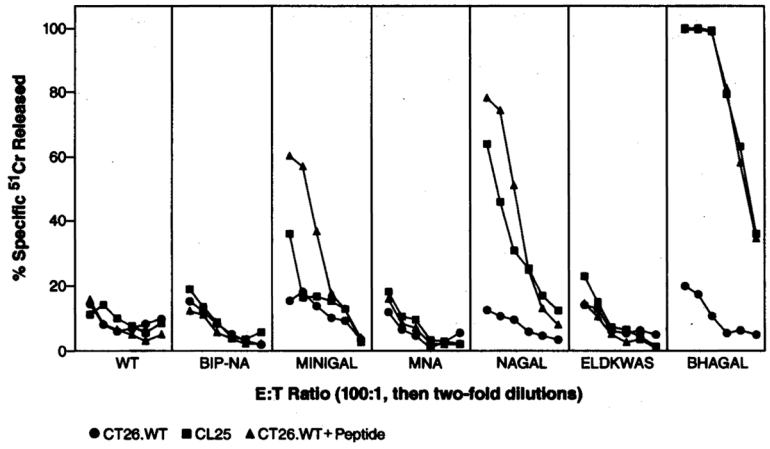

FIG. 3.

Specific cytolytic responses induced in mice by transfectant influenza A viruses expressing the β-gal epltope TPHPARIGL To evaluate the function of transfectant influenza A viruses in the priming of β-gal-specific cytotoxic responses *in vivo*, two mica/group were injected intravenously with 10^6 PFU of the influenza A virus shown on the abscissa. Three weeks later, splenocytes from immunized mice were cultured in the presence of the L^d-restricted β-$gal_{876–884}$ peptide for 6 days, then tested in a microcytotoxicity assay against CT26.WT, CT26.CL25, or CT26.WT cells loaded with the β-$gal_{876–884}$ peptide at the indicated E:T ratios. Experiment was repeated with similar results.

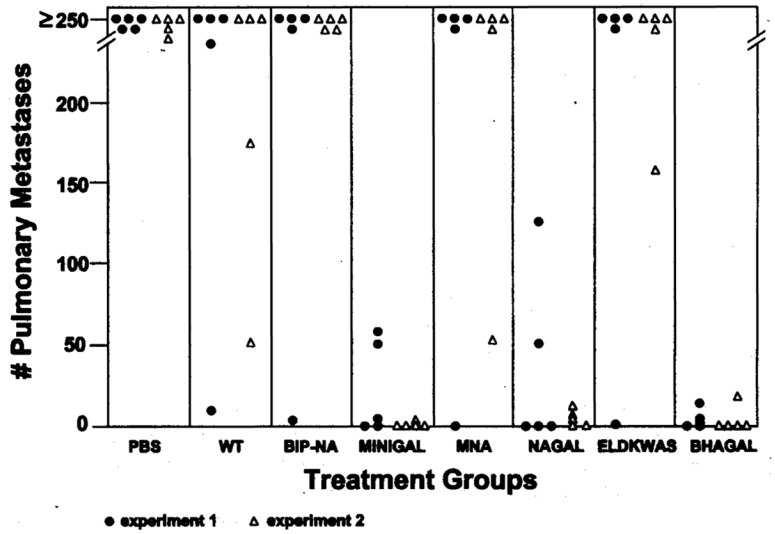

FIG. 4.

Transfectant influenza A viruses mediate treatment of pulmonary metastases established for 3 days. Mice were inoculated intravenously with 5×10^6 CT26.CL25 tumor cells, then vaccinated Intravenously 3 days later with 10^6 PFU of the transfectant influenza A virus shown. The lungs of treated mice were evaluated In a coded, blinded manner for pulmonary metastases 12 days after the tumor inoculation. The number of pulmonary metastases that were enumerated after two independent experiments are shown for individual mice.

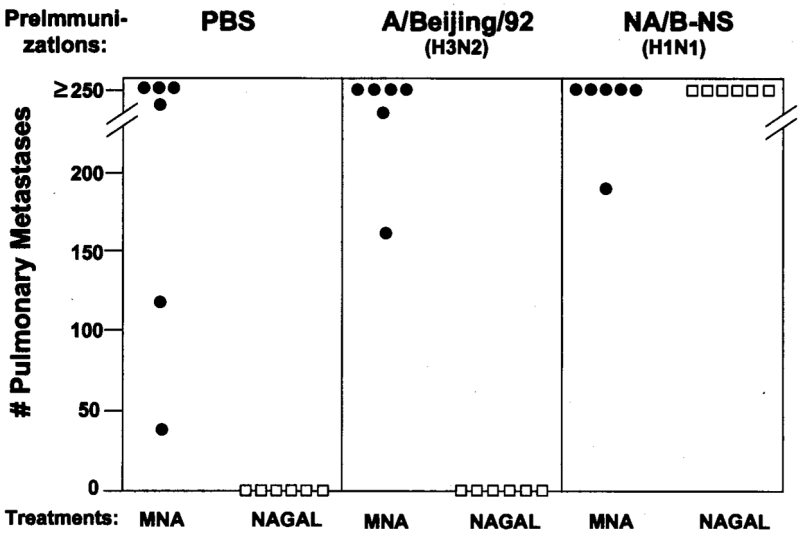

FIG. 5.

Transfectant NAGAL influenza virus (H1N1) mediates regression of established pulmonary metastases in mice that were preimmunized with H3N2 influenza A virus. Twelve mice were inoculated intranasally with PBS, 10^3 PFU of influenza NA/B-NS (H1N1) virus or 10^3 PFU of influenza A/Beijing/32/92 (H3N2) virus. One month after immunization, 5×10^6 CT26.CL25 tumor cells were inoculated intravenously. Three days after tumor cell inoculation, the mice were injected intraperitoneally with 10^6 PFU of MNA (dark circles) or NAGAL (open squares) transfectant viruses. The number of pulmonary metastases was determined 12 days after tumor inoculation.