Intracellular antibody-caspase-mediated cell killing: An approach for application in cancer therapy

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Antibodies have been expressed inside cells in an attempt to ablate the function of oncogene products. To make intracellular antibodies more generally applicable and effective in cancer therapy, we have devised a method in which programmed cell death or apoptosis can be triggered by specific antibody–antigen interaction. When intracellular antibodies are linked to caspase 3, the “execu
tioner” in the apoptosis pathway, and bind to the target antigen, the caspase 3 moieties are self-activated and thereby induce cell killing. We have used this strategy in a model system with two pairs of intracellular antibodies and antigens. In vivo coexpression of an antibody-caspase 3 fusion with its antigenic target induced apoptosis that was specific for antibody, antigen, and active caspase 3. Moreover, the antibody-caspase 3 fusion protein was not toxic to cells in the absence of antigen. Therefore, intracellular antibody-mediated apoptosis should be useful as a specific therapeutic approach for the treatment of cancers, a situation where target cell killing is required.

Cancer is characterized by mutations in oncogenes and by chromosomal translocations, which give rise to enforced expression of oncogenes or chimeric fusion proteins (1). The protein products of these abnormal genes are unique to cancer cells and are therefore tumor-specific antigens. Although such proteins are potential targets for therapeutic intervention, they are mainly intracellular proteins and therefore present practical difficulties in the design of therapeutic strategies. One approach is the intracellular expression of antibodies or fragments, in particular single-chain Fv (scFv), to inactivate mutant proteins either by directly neutralizing their functions (2) or by preventing them from reaching the necessary cell compartments (3). However, this strategy relies on neutralizing effects of the scFv and therefore on the function of the target proteins. A more effective use of intracellular antibodies in cancer gene therapy would be to induce tumor cell killing—for example, taking advantage of the programmed cell death or apoptosis pathway—after recognition of the target antigen.

Apoptosis is a process in which living cells undergo programmed death, triggered by various external or internal stimuli (4, 5). The process is tightly regulated because the loss of cells within an organism must be controlled for viability. Apoptosis is carried out by a family of cysteine proteases known as caspases that cleave at specific amino acids (6). One member of the family, caspase 3, is the so-called executioner in apoptosis and is responsible for the cleavage of many proteins important in maintaining the integrity of living cells (7). It is synthesized as zymogen and is cleaved and activated by the initiator or upstream caspases such as caspase 8 to form an active tetrameric enzyme (8). Although there is no evidence of autoactivation of caspase 3 under physiological conditions, it has been shown that forced dimerization of two molecules can cause self-activation and irreversibly lead to cell death (9, 10). Thus if two caspase 3 molecules can be brought close enough together, cell death should ensue.

We have developed a method of cell killing that is based on the activation of caspase 3 by means of intracellular antibody–caspase 3 fusion. We describe a model system in which the autoactivation of caspase 3 occurs after specific intracellular antibody–antigen binding. Using an anti-β-galactosidase (β-gal) antibody that binds to antigen in vivo but does not neutralize its enzyme activity (11), we can demonstrate antibody-, antigen-, and caspase-specific cell killing.

Materials and Methods

Construction of Expression Plasmids. pM-βgal, pNL-scFvR4-VP16, pNL-scFvF8-VP16, and pNL-scFv-IN33-VP16 were described previously (12). pRSV-Luc [firefly luciferase expression vector] was also described previously (13) and pEGFP-N1 [enhanced green fluorescence protein (GFP) expression vector] was commercially available (CLONTECH).

The pEF-βgal (β-gal expression vector). This vector was created by subcloning the coding sequence of β-gal and simian virus 40 poly(A) from pBSpt-βgal (14) into the pEF-BOS mammalian expression vector (15).

The shuttle vectors pBS-R4 and pBS-F8. These vectors were made by cloning the Clal–EcoRI fragment of pNL-scFvR4-VP16 and pNL-scFvF8-VP16, respectively, into pBSpt.

The pEF-R4-DBD (scFvR4-GAL4DBD fusion expression vector). GAL4 DNA-binding domain (DBD) sequence [PCR amplified from pGALO (16)] was cloned in-frame with the scFvR4 and therefore on the function of the target proteins. A more effective use of intracellular antibodies in cancer gene therapy would be to induce tumor cell killing—for example, taking advantage of the programmed cell death or apoptosis pathway—after recognition of the target antigen.

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We have developed a method of cell killing that is based on the activation of caspase 3 by means of intracellular antibody–caspase 3 fusion. We describe a model system in which the autoactivation of caspase 3 occurs after specific intracellular antibody–antigen binding. Using an anti-β-galactosidase (β-gal) antibody that binds to antigen in vivo but does not neutralize its enzyme activity (11), we can demonstrate antibody-, antigen-, and caspase-specific cell killing.

Abbreviations: scFv, single-chain Fv; β-gal, β-galactosidase; GFP, green fluorescent protein; DBD, DNA-binding domain; FACS, fluorescence-activated cell sorting.

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respectively, pEF-IN33-CP3 and pEF-IN33-CP3(C163S) were constructed by cloning the XhoI–SpeI fragment of pBS-IN33-CP3 and pBS-IN33-CP3(C163S) into pEFBOS. pEF-HIVIN-βgal [HIV integrase (amino acids 259–288)]-β-gal fusion expression vector). The NcoI–SalI fragment of pBluescript-βgal was subcloned into pEF/myc/cytO (Invitrogen) and the PCR-amplified HIV-1 integrase epitope (amino acids 259–288) was cloned as a NcoI fragment in-frame at the 5’ end of the β-gal sequence to give pEF-HIVIN-βgal.

Mammalian Cell Culture and Transfection. Chinese hamster ovary (CHO) cells were grown in α MEM with 10% FCS, penicillin, and streptomycin. CHO cells (2 × 10⁶) were seeded onto a 35-mm Petri dish 16–24 h before transfection. Transfection was performed by using Lipofectamine (GIBCO/BRL) with 500 ng of pEF-βgal, pEF-HIVIN-βgal, and pRsv-Luc, 50 ng of pEGFP-N1, and 250 ng of pEF-scfv-Cp3/CP3(C163S), except pEF-IN33-CP3/CP3(C163S), for which 50 ng was used. Cells were harvested 60 h after transfection.

The CHO-CD4 line has been reported previously (17). It was maintained with α MEM, 10% FCS, and 1 mg/ml G418 (GIBCO/BRL). Lipofectamine transfection of CHO/CD4 cells growing on 100-mm dishes at 50–60% confluence was performed with 5 μg of each plasmid unless stated otherwise.

Fluorescence-Activated Cell Sorting (FACS) Analyses for CD4 Expression. CHO-CD4 cells were harvested 48 h after transfection. CD4 expression was analyzed by binding a mouse anti-human CD4 antibody (PharMingen) at 1:50 dilution and a secondary FITC-conjugated goat anti-mouse polyclonal antibody (PharMingen) at 1:100 dilution. The relative fluorescence of the cells was measured with a FACS Calibur instrument (Becton Dickinson).

Western Blotting. CHO cells were transfected with scFvR4-caspase3, scFvR4-caspase3(C163S), and scFvF8-caspase3 expression vectors. Forty-eight hours after transfection, cells were lysed in 10 mM Hapes, pH 7.6/250 mM NaCl/5 mM EDTA/0.5% Nonidet P-40. The lysates were fractionated by SDS/12% PAGE and transferred to nitrocellulose membrane. The membrane was incubated with anti-human caspase 3 antibody (Santa Cruz Biotechnology) and a secondary horseradish peroxidase (HRP)-conjugated anti-goat antibody (Santa Cruz Biotechnology). Detection is performed with ECL Western blotting detection reagents (Amersham). For the detection of the 17-kDa cleaved caspase 3 fragment, a caspase 3-specific (cleaved caspase specific) rabbit polyclonal antibody (New England Biolabs) was used, followed by secondary HRP-conjugated anti-rabbit IgG antibody (Amersham).

β-gal and Luciferase Activity Assays. Transfected CHO cells were lysed with 300 μl of Reporter Lysis Buffer (Promega) per 35-mm Petri dish at the specified times after transfection. β-gal activities were measured by using β-gal Enzyme Assay System (Promega) according to the supplier’s instructions. Luciferase activity measurements were performed using Luciferase Assay System (Promega) and a luminometer. Two separate independent transfections were done and the averaged result is presented.

Apoptosis Assay. CHO cells were cotransfected with GFP expression vector pEGFP-N1, the various scFv fusion expression vectors, and with or without pEF-βgal. Thirty-six hours after transfection, GFP-positive CHO cells were sorted by FACS. Genomic DNA was extracted from approximately 5,000 sorted cells. The presence or absence of nuclear DNA products was determined by using the ApoAlert LM-PCR Ladder Assay Kit (CLONTECH). Genomic DNA was amplified by using the ApoAlert primers, which amplify DNA generated from the chromatin beads resulting from apoptosis. PCR products were visualized by ethidium bromide staining after fractionation on 1.5% agarose gels. As a control for DNA yield in each set of FACS-sorted cells, primers specific for Chinese hamster actin were used (5’-GGCGTGATGGTGCCATGCGCC-3’ and 5’-CTGGTCACTTTTTACGGTTCG-3’) for PCR. The PCR consisted of 35 cycles of 94°C denaturing for 1 min, 65°C annealing for 1 min, and 72°C extension for 1 min. The products were also visualized on 1.5% agarose gels.

Results

Activation of Transcription by Proximity of Anti-β-gal scFv-VP16 Bound to Antigen. The crystal structure of β-gal shows that a tetramer is necessary for enzyme activity (18). Therefore antibodies that bind to β-gal do so at four separate antigenic sites per active enzyme. An anti-β-gal scFv has been described, scFv-R4, that binds to β-gal in both bacterial (11) and mammalian cells (12) but does not affect β-gal function. This model intracellular antibody system was used to determine whether proximity of scFv antibody fragments in vivo would cause measurable effects. For this assessment, we initially undertook to determine whether transcriptional transactivation could be mediated by scFv-R4 binding to antigen. Our assay consisted of coexpression of β-gal with scFv-R4 fused to a GAL4 DBD and scFv-R4 fused to the VP16 transcriptional transactivation domain (AD), in a CHO cell line with a CD4 reporter gene controlled by the GAL4 promoter (17). The CHO-CD4 cells will express CD4 on their cell surface if the reporter gene is activated. Therefore, if the intracellular antibody scFv-R4-DBD and scFv-R4-VP16 bind to β-gal in CHO-CD4 cells, a transcription complex should be created that causes activation of the CD4 gene (illustrated in Fig. 1).

Various plasmids were cotransfected in CHO-CD4 cells and, 60 h after transfection, cell surface CD4 was measured by FACS analyses (Fig. 1B). When β-gal was directly linked to the GAL4-DBD, expression of scFv-R4-VP16 fusions results in efficient CD4 surface expression (panel 2), but scFv-R4 fused to GAL4 DBD or to VP16 failed to activate CD4 in the absence of β-gal (panel 3). Therefore the DBD-β-gal fusion can interact with the DNA-binding sites of the CD4 reporter and a transcription complex is created when scFv-R4 linked to VP16 activation domain also binds to the β-gal epitopes on the DBD-β-gal tetramer.

Next we expressed β-gal with scFv-R4 fused to the DBD or fused to the VP16 transactivation domain to assess the formation of a DNA-binding complex. For this to be effective, both scFv-DBD and scFv-VP16 must bind to different sites on the same β-gal tetramer. We therefore titrated different amounts of expression vectors to alter the ratio of scFv-DBD and scFv-VP16 fusion proteins (Fig. 1B, panels 4–9). CD4 reporter gene activation was observed in all cases but less efficiently than the DBD-β-gal coexpression (the percentage of cells expressing CD4 in these transient assays ranging from 0.24% to 2.8%). The relative inefficiency presumably reflects the bulkiness of the protein complex in the transcription assay and the need for multiple binding sites on each β-gal. The degree of CD4 expression depended on the ratio between scFvR4-VP16 and scFvR4-DBD (Fig. 1B, panels 6, 7, and 8) because the two different scFvR4 fusion proteins will compete for the same binding sites on the β-gal tetramer. It was confirmed that no detectable dimerization occurs between scFvR4 because CD4 activation did not occur when the scFvR4-VP16 and scFvR4-DBD were expressed without the antigen (Fig. 1B, panel 3). We conclude that protein domains that are linked to scFvR4 can be brought into sufficiently close proximity for biochemical interactions when the scFvR4 binds to β-gal epitopes in vivo.

ScFv-Caspase 3 Fusion Causes Apoptosis After Binding to Antigen. The data presented above suggested that the molecular distance
Whereas b follows:

CD4 reporter gene. (chromosomal GAL4 indicated. The amount of pEF-

12268 autoactivation and triggering of apoptosis (illustrated in Fig. 2

b-gal tetramer, can form a transcription complex that can bind to the chromosomal GAL4 DNA-binding site (DBS) and controls transcription of the CD4 reporter gene. (b) CHO-CD4 cells were cotransfected with b-gal expression clone pEF-Jigal, together with various expression vectors. Induction of cell surface CD4 expression was assayed after 60 h by using anti-human CD4 antibody. The indicated percentage of CD4+ cells after 48 h was estimated with a FACScanibur machine. The coexpressed vectors with pEF-Jigal were as follows: 1, pEF-Bos vector only; 2, DBD-Jigal and scFvR4-VP16; 3, scFvR4-VP16 and scFvR4-DBD; and 4–5, various amounts of scFvR4-VP16 and scFvR4-DBD as indicated. The amount of pEF-Jigal plasmid (5 µg) was not varied.

between the scFv-R4 antigenic sites might be small enough to bring the caspase 3 moieties close enough together to cause autoactivation and triggering of apoptosis (illustrated in Fig. 2A). An assessment of this was made by cotransfecting CHO cells with the reporter b-gal expression plasmid along with various scFv-R4 expression clones, including one encoding a fusion of scFvR4 with pro-caspase 3. Expression of each of the scFv fusion proteins was confirmed by using anti-caspase 3 antibody in Western blots of protein extracts from CHO cells transfected with the expression vectors (Fig. 2B). Sixty hours after transfection, the cells were assayed for b-gal activity (Fig. 2C). Whereas b-gal was detected in the control transfection (column 1), when the scFv-R4 was linked to caspase 3, we observed very little b-gal activity (column 3). This loss of b-gal activity was dependent on the activity of caspase 3, as judged by the effect of an inactivating caspase 3 mutation (9) in which the catalytic cysteine was mutated to a serine (C163S, column 4). In addition, no significant difference was observed when the b-gal reporter was expressed with scFv-R4 fused to VP16 (column 2). The antibody specificity causing the lack of b-gal activity was shown by cotransfecting a nonspecific scFv [scFv-F8 (19)] fused with caspase 3. This combination had no effect on b-gal levels. These data therefore suggest that the reduction in b-gal activity when scFv-R4-caspase 3 is cotransfected is attributable not to a neutralizing effect on b-gal but rather to the proteolytic activity of the activated caspase 3, causing apoptosis of the transfected cells. It is also of note that scFv-caspase 3 fusion alone is not toxic to cells (column 5), which is consistent with previous reports (9, 20).

Induction of cell killing by the scFvR4-caspase 3 fusion molecule, dependent on the interaction of specific scFv-caspase 3 and b-gal antigen, was confirmed by using an independent reporter to which the scFvR4 antibody does not bind. CHO cells were transfected with such a reporter, pRSV-Luc, constitutively expressing firefly luciferase, together with either scFvR4-caspase 3, scFvR4-VP16, mutant scFvR4-caspase 3 (C163S) or scFvR4-caspase 3 in the presence or absence of b-gal expression (i.e., scFv-R4 antigen). Luciferase activity was measured 60 h after transfection, as a measure of cell viability in the presence of scFv fusion proteins (Fig. 2D). These data show about 80% decrease in luciferase activity when scFvR4-caspase 3 was expressed along with b-gal (column 3, front row). However, this lack of luciferase activity in cells transfected with scFv-R4-caspase 3 would appear to be due to antigen-dependent cell death, rather than toxicity of the expressed scFv-R4-caspase 3 alone, as no loss of viability was observed when scFvR4-caspase 3 was expressed without b-gal (column 3, back row). The caspase-dependence is shown by the scFvR4-caspase mutant protein fusion, which has no reduction in luciferase either in the presence or in the absence of b-gal expression. Finally, antibody specificity was confirmed by using the nonspecific scFv-F8, which does not affect reporter gene activity. These results indicate that induced apoptosis was antibody and antigen specific, and dependent on caspase 3 activity.

To show that there is specific cleavage of the pro-caspase 3 moiety caused by activation of the scFvR4-caspase 3 fusion upon binding to b-gal, Western blotting was performed to detect the cleaved caspase 3 fragment. Forty-eight hours after transfecting CHO cells with scFvR4-caspase 3 fusion, with and without b-gal, the 17-kDa fragment of activated caspase 3 was detectable only when scFvR4-caspase 3 was expressed in conjunction with b-gal (Fig. 2E). The amount of protein loaded in each lane was comparable as judged by immunodetection of actin protein (Fig. 2F). This result confirmed that activation of caspase 3 occurred after specific antigen–antibody interaction in vivo.

While our results in the b-gal and luciferase assays show a dependence on active caspase 3, this provides indirect evidence that scFv-caspase 3 causes apoptosis after binding to antigen. Direct evidence for apoptosis mediated by scFv-caspase 3 was obtained by studying DNA from transfected cells to assess the presence of a chromatin bead ladder, a hallmark of apoptotic cell death caused by nuclease digestion of chromatin (21). CHO cells were transfected with a marker plasmid, pEGFP-N1, expressing GFP, together with plasmids expressing the various scFv fusion proteins, and with or without the b-gal expression vector. After 36 h, transfected cells that expressed GFP (therefore also scFv-R4 and b-gal) were enriched by using FACS. Genomic DNA was extracted from these sorted cells and DNA fragments, emanating from the apoptosis-mediated chromatin digestion, were assessed by the ligation-mediated PCR procedure (22) (Fig. 3A). We found evidence of chromatin beads only in the DNA prepared from cells cotransfected with scFvR4-caspase 3 and b-gal (lane 2) and not in those transfected with b-gal and scFvR4-VP16 (lane 1), scFvR4-caspase 3(C163S) (lane 3), or scFvR8-caspase
3 (lane 4). Yield of DNA in each was comparable as determined by PCR using actin gene primers (Fig. 3B). Moreover, scFvR4-caspase 3 transfected in the absence of β-gal did not generate the DNA ladder (lane 5), indicating that the apoptosis depended on the specific interaction between the intracellular antibody-caspase fusion (scFvR4-caspase 3) and antigen (β-gal).

**General Applicability of Intracellular Antibody-Mediated Apoptosis.** To consolidate the general applicability of the intracellular antibody-mediated apoptosis approach, a second system was developed using a different antigen and intracellular antibody pair, namely a small antigenic epitope of HIV-1 integrase and specific antibody recognizing this epitope in vivo (scFvIN33) (23). The structure of β-gal (18) predicts that any protein linked to the N terminus of β-gal monomer will be positioned at the interface of the tetrameric β-gal molecule. As a result, the physical distance between the linked moieties should be short, as will the distance between the specific antibodies that bind to them. Therefore, an expression construct, pEF-HIVIN-β-gal, was made in which the HIV-1 integrase amino acids 259–288 [i.e., those recognized by scFvIN33 (24)] were fused at the N terminus of β-gal. This vector was coexpressed in CHO cells with a scFvIN33-caspase 3 fusion protein. Interaction of antibody and antigen should cause caspase-mediated apoptosis as depicted in Fig. 4A and β-gal activity would reflect cell viability measured at 60 h after transfection.

In parallel with our findings with anti-β-gal scFv, the expression of β-gal was markedly decreased when the HIV-integrase-β-gal fusion was coexpressed with scFvIN33-caspase 3 (Fig. 4B, column 2, front row) compared with the HIV-integrase-β-gal fusion alone (column 1, front row). Furthermore, expression of scFvIN33-caspase 3 with wild-type β-gal did not result in significant reduction in β-gal activity (Fig. 4B, column 2, back row), indicating that cell death had occurred in response to dimerization of scFv-caspase 3 after binding to sites on the HIV-integrase-β-gal fusion (as illustrated in Fig. 4A) and not because of autotoxicity of scFvIN33-caspase 3. The requirement for active caspase 3 was demonstrated by using a mutant scFv-caspase, scFvIN33-caspase 3(C163S) (column 3), and the antibody specificity was shown by using the nonspecific antibody.
scFvR4-caspase 3, caused cell death in cells expressing wild-type scFvR4-caspase 3 (C163S). The procedure utilizes the normal process of programmed cell death coupled to a cell death function if appropriately modified. We reagents that can bind to target antigens (11, 26). Intracellular antibodies or fragments are important binding capability inside mammalian cells (12). Alternatively, such as an antibody, because they fold incorrectly as the critical disulfide bonds cannot be formed in the reducing environment of cytoplasm (25). Presumably, they fold incorrectly as the critical disulfide bonds (11)-gal levels, whereas the anti-β-gal antibody fusion, scFvR4-caspase 3, caused cell death in cells expressing wild-type β-gal and HIV integrase β-gal fusion as expected (column 4, back and front rows). Cell death in this model system is, therefore, antigen specific, antibody specific, and dependent on active caspase 3.

**Discussion**

Intracellular scFv have been used to bind to oncogene products in tumor cells preventing the cells from displaying the tumor phenotype (2, 3). However, the tumor phenotype can be caused by more than a single mutation, and thus ablation of the function of one protein will not necessarily be effective. In addition, in the clinical setting, epithelial cancers tend to become disseminated and thus therapies that rely on cell killing, rather than merely blocking protein function, are likely to be more effective.

There are two major obstacles for the potential use of intracellular antibodies in cancer gene therapy. First, most antibodies do not bind to their antigens inside mammalian cells. Presumably, they fold incorrectly as the critical disulfide bonds cannot be formed in the reducing environment of cytoplasm (25). Second, even if a particular antibody retains its binding ability and specificity in vivo, it may not neutralize the function of the protein, and hence has no effect on the phenotype of the cancer cells (2). The first problem can be overcome by using selection methods that directly identify intracellular binders, such as an in vivo two-hybrid system for selecting antibodies with binding capability inside mammalian cells (12). Alternatively, intracellular binders can also be isolated by molecular evolution (11, 26). Intracellular antibodies or fragments are important reagents that can bind to target antigens in vivo and could be coupled to a cell death function if appropriately modified. We devised a method in which direct cell killing can be induced by intracellular antibody–antigen interaction in tumor cells. Our procedure utilizes the normal process of programmed cell death/apoptosis and takes advantage of the fact that caspase 3, a major component in the process of apoptosis, can undergo autoactivation when two molecules are brought in close proximity (9). By exploiting this mechanism, we showed that when two or more scFv-caspase 3 fusion proteins bind to the epitopes of an antigen that are close together, the caspase 3 moieties can autoactivate when two molecules are brought in close proximity (9). By exploiting this mechanism, we showed that when two or more scFv-caspase 3 fusion proteins bind to the epitopes of an antigen that are close together, the caspase 3 moieties can autoactivate when two molecules are brought in close proximity (9). By exploiting this mechanism, we showed that when two or more scFv-caspase 3 fusion proteins bind to the epitopes of an antigen that are close together, the caspase 3 moieties can autoactivate when two molecules are brought in close proximity (9).
by the specific binding between intracellular antibody-caspase 3 fusion proteins and the respective antigen. In each case, the cells specifically expressing target antigen were killed, demonstrating the general applicability and specificity of this intracellular antibody-mediated cell killing.

There are several types of tumor cells that can be attacked by the scFv-caspase 3 approach. The first is analogous to the antibody-mediated cell killing. Specifically expressing target antigen were killed, demonstrating fusion proteins and the respective antigen. In each case, the cells by the specific binding between intracellular antibody-caspase 3.

Moreover, apoptosis does not provoke any harmful inflammatory reaction. All these advantages should make the approach useful in the field of disease therapy. The derivation of intracellular scFv linked to caspase 3 is nontoxic to cells and, because the induced cell killing takes place in a few hours and is irreversible, a single mutation and another scFv that binds to a common site in the apoptotic cascade for caspase effectors. However, caspase 8 was tested in our model systems, but we found it had more antigen-independent autoactivation (data not shown), making it unsuitable for use in this approach, at least in its wild-type state. Future use for cases of low target antigen concentration could employ mutated caspase 8 with higher threshold of autoactivation, or mutated caspase 3 with lower threshold of autoactivation. This modifications may need to be implemented if levels of antigen and/or levels of activation of apoptosis require it. In any event, the proof-of-principle experiments described here illustrate a general strategy for killing target cells through the presence of specific antigens.

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