Redirecting anti-viral CTL against cancer cells by surface targeting of monomeric MHC class I-viral peptide conjugated to antibody fragments

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Abstract

To combine the advantage of both the tumor targeting capacity of high affinity monoclonal antibodies (mAbs) and the potent killing properties of cytotoxic T lymphocytes (CTL), we investigated the activity of conjugates made by coupling single Fab' fragments, from mAbs specific for tumor cell surface antigens, to monomeric HLA-A2 complexes containing the immunodominant influenza-matrix peptide 58-66. In solution, the monovalent 95 kDa Fab-HLA-A2/Flu conjugates did not activate influenza-specific CTL. However, when targeted to tumor cells expressing the relevant tumor-associated antigen, the conjugates induced CTL activation and efficient tumor cell lysis, as a result of MHC/peptide surface oligomerization. The highly specific and sensitive in vitro cytotoxicity results presented suggest that injection of Fab-MHC/peptide conjugates could represent a new form of immunotherapy, bridging antibody and T lymphocyte attack on cancer cells.

Introduction

The efficiency of T cell-based active immunotherapy (1, 2, 3) may be limited by the absence or low expression of either MHC molecules (4, 5) or their associated antigenic peptides by tumor cells (6). In contrast, monoclonal antibodies binding with high affinity to cell surface molecules abundantly expressed on cancer cells have been shown in clinical studies to localize specifically to tumor tissues (see 7 for review). Furthermore, injections of large amounts of selected mAbs have proven to be useful in the treatment of B cell lymphoma and breast carcinoma (8, 9). However, the capacity of antibodies to kill carcinoma cells is much weaker than that of cytotoxic T lymphocytes and clinical trials based on the use of antibodies alone showed less than 10% of complete tumor remission (8, 9).

Thus, the idea of using mAbs reacting with tumor cells to target CTL-defined antigens in the form of Fab' fragments coupled to a soluble MHC/peptide complex represents an attractive immunotherapeutic strategy. As a first step in that direction, we recently showed that MHC class I/peptide tetramers assembled on streptavidin molecules that were coupled to tumor-reactive antibody Fab' fragments induced a specific tumor cell killing by relevant CTL (10). Lysis was strictly dependent on expression of the target antigen by the tumor cells. In a three step strategy, it was also recently shown that the sequential coating of tumor cells with biotinylated anti-tumor antibodies, followed by streptavidin and biotinylated MHC/peptide complexes can also sensitize tumor cells for killing by CTL (11).
In this report, we demonstrate that antibody-dependent tumor cell sensitization to CTL-mediated lysis does not require tetramerization of MHC/peptide complexes, which is essential for cytofluorometric analysis of T-lymphocyte responses (12). Indeed, monomeric MHC/peptide complexes coupled to an Fab' fragment from a selected tumor-reactive mAb can be targeted at sufficient density on the surface of tumor cells to induce the affinity enhancement required for triggering specific CTL-mediated killing of tumor cells. As all the Fab-MHC/peptide complexes targeted on tumor cells present the same antigenic peptide, such a procedure greatly increases the efficiency of CTL activity.

As a model system, we used the immunodominant HLA-A2 restricted CTL epitope derived from the matrix protein of the influenza virus (HLA-A2/Flu). Indeed, this epitope is representative of a strong and frequent anti-viral CTL response, since most HLA-A2 positive individuals, including cancer patients have circulating memory CTL against this epitope, as demonstrated ex vivo by tetramer staining (13). As tumor targeting antibodies, we selected Fab' fragments derived from mAbs already used in clinical trials (8, 9, 14) and which are directed against antigens such as ErbB-2, CD20 and carcinoembryonic antigen (CEA) (subsequently referred to as tumor-associated antigens).

To obtain MHC/peptide complexes coupled to Fab' fragments, we first produced soluble recombinant MHC/peptide complexes following established methods (15), except that we introduced by point mutation a single cysteine residue near the C-terminal end of the MHC heavy chain. This free thiol group was used for coupling the complexes, via a bismaleimide spacer, to a free cysteine residue from Fab' antibody fragments. We expect, and already have preliminary evidence, that the resulting 95 kDa conjugates will be of optimal size for tumor targeting in vivo, since we have previously shown, both in nude mice and in patients, that radiolabeled anti-CEA F(ab')2 fragments of similar size (100 kDa) have excellent tumor targeting properties (14, 16). As shown here, when coupled to monomeric HLA-A2/Flu peptide complexes, Fab' fragments directed against three different tumor-associated antigens induced specific CTL-mediated lysis of tumor cells very efficiently.

### Results

**Synthesis and characterization of Fab-HLA-A2/peptide conjugates**

Soluble recombinant HLA-A2 molecules containing a cysteine introduced by site directed mutagenesis at position 275 of the C-terminal end of the heavy chain were produced in bacteria. Monomeric complexes of HLA-A2 heavy chain, beta2-microglobulin (beta2M) and synthetic influenza matrix (Flu-MA) peptide58-66 were folded by the dilution method and purified as described (12, 13, 15). The free thiol group of cysteine 275 was then derivatized with an excess of the bismaleimide polyethylene oxyde linker (Fig. 1A). After removal of the free linker, the maleimide-derivatized HLA-A2/peptide complexes were coupled by a thioether bond to the free cysteine of Fab' fragments, obtained by reduction of F(ab')2 fragments from different tumor reactive mAbs, including murine anti-CEA (14, 16), humanized anti-ErbB-2 (17) and chimeric anti-CD20 (18). Following filtration on a Superdex 200 FPLC column, the conjugates were eluted with an apparent Mr of 95 kDa. The coupling efficiency was approximately 30% (Fig. 1B). In SDS-PAGE under nonreducing conditions, the conjugates showed a major band with an apparent Mr of 82 kDa, due to dissociation of beta2M and peptide, while under reducing conditions, a single band of approximately 57 kDa was obtained corresponding to thioether-linked HLA-A2 heavy chain and pepsin cleaved Fab' heavy chain (Fig. 1C).

**Specific coating of tumor cells by Fab-HLA-A2 conjugates demonstrated by cytofluorometry**

The Fab-HLA-A2/Flu conjugates specific for CEA, ErbB-2 and CD20 were tested for their capacity to bind to three HLA-A2 negative cell lines: the CEA positive colon carcinoma cell LoVo, the ErbB-2 positive breast carcinoma line SK-BR-3 and the CD20 positive B cell lymphoma line Daudi. Figure 2 shows the positive cytofluorometry profiles obtained with an FITC-labeled anti-HLA-A2 mAb (6) on LoVo, SK-BR-3 and Daudi cells preincubated with anti-CEA, anti-ErbB-2 and anti-CD20 Fab-HLA-A2 conjugates, respectively. Negative controls were cells without conjugate. These results demonstrate the capacity of Fab-HLA-A2/Flu conjugates to specifically bind to cells expressing the relevant tumor-associated antigen.
Figure 1. Schematic diagram of the synthesis and characterization of a chemical conjugate between an antibody Fab' fragment and a recombinant MHC class I, beta2M, peptide complex. (A) Purified HLA-A2/Flu-MA peptide complexes containing a heavy chain substituted with a cysteine residue near the C-terminus were first derivatized with a bismaleimide polyethylene oxide linker and then coupled to the reduced cysteine of a purified Fab' fragment. (B1) Elution profile from a Superdex 200 FPLC column of the purified HLA-A2/Flu peptide complex. (B2) Purification of the Fab-HLA-A2/Flu conjugate with a yield of approximately 30%. (B3) Elution profile of the purified Fab-HLA-A2/Flu conjugate with an apparent Mr of approximately 95 kDa. (C) SDS-PAGE showing that the purified Fab-HLA-A2/Flu conjugate gives under nonreducing conditions a major band with an apparent Mr of 82 kDa (lane 3), while as control proteins, intact mAb gives a band of 150 kDa (lane 1) and the F(ab')2 fragment a band of 100 kDa (lane 2). Under reducing conditions, the conjugate gives a single band with an apparent Mr of 57 kDa (lane 6) while intact mAb (lane 4) gives two bands of 50 and 25 kDa and F(ab')2 fragment a band of 25 kDa (lane 5).

Specific CTL-mediated lysis of Fab-HLA-A2 conjugates-coated tumor cells

Four tumor cell lines expressing different tumor-associated antigens preincubated with each of the specific Fab-HLA-A2/Flu peptide conjugates were efficiently lysed by HLA-A2-restricted, Flu-MA peptide-specific CTL as assessed in a 51Cr release assay (Fig. 3). Precoating of CEA positive LoVo cells with anti-CEA Fab-HLA-A2/Flu conjugates induced their lysis by the relevant CTL, while the same conjugates did not induce lysis of the CEA negative SK-BR-3 cells (Fig. 3A). Similarly, anti-ErbB-2 and anti-CD20 Fab-HLA-A2/Flu conjugates induced specific lysis of the ErbB-2 positive SK-BR-3 and the CD20 positive Daudi and Raji cell lines, respectively (Fig. 3B and C). In terms of efficiency, >25% specific lysis was already detectable at an effector to target cell ratio (E/T) of 1/1 and 75% specific lysis was reached at an E/T ratio of 20/1, in the presence of 2 µg of conjugate per ml (Fig. 3). The low percent lysis (approx. 20%) of LoVo cells preincubated with anti-ErbB-2 Fab-HLA-A2/Flu conjugate (Fig. 3B) is likely due to the presence of low levels of ErbB-2 molecules on these cells (19), since lysis could be inhibited by unconjugated anti-ErbB2 mAb (see below).

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Figure 2. Cytofluorometry analysis demonstrating the specific coating of anti-tumor Fab-HLA-A2/Flu conjugates on the surface of HLA-A2 negative tumor target cells, using a FITC-labeled anti-HLA-A2 mAb. (A) CEA positive colon carcinoma cells LoVo. (B) ErbB-2 positive breast carcinoma cells SK-BR-3. (C) CD20 positive B lymphoma cells Daudi were incubated with anti-CEA Fab-HLA-A2, anti-ErbB-2 Fab-HLA-A2 or anti-CD20 Fab-HLA-A2 conjugates respectively, followed by FITC-labeled anti-HLA-A2 (thick solid histograms) or with the latter conjugate alone (grey shaded histograms).

Figure 3. Demonstration of the specific lysis of cancer cells coated with different Fab-HLA-A2/Flu peptide conjugates, by a relevant CTL clone at different effector to target cell ratios. (A) Specific lysis, in a $^{51}$Cr release assay, of LoVo colon carcinoma target cells preincubated with anti-CEA Fab-HLA-A2/Flu (filled squares) and no lysis of the CEA negative SK-BR-3 cells (open circles) preincubated with the same conjugate. (B) Specific lysis of SK-BR-3 induced by anti-ErbB-2 Fab-HLA-A2/Flu (filled circles) with minimal lysis of LoVo cells (open squares) or Daudi cells (open diamonds) preincubated with the same conjugate. (C) Specific lysis of Daudi (filled diamonds) and Raji (crosses) cells preincubated with anti-CD20 Fab-HLA-A2/Flu with no lysis of LoVo (open squares) or SK-BR-3 (open circles) cells preincubated with the same conjugate. In all panels, the conjugate concentration was 2 µg/ml. Results are expressed as % specific target cell lysis.
Conjugate titration and specific inhibition by competing antibody

The HLA-A2 negative tumor cell lines expressing different tumor-associated antigens, as described above, were incubated for 1 h at 37°C with increasing concentrations of each of the three Fab-HLA-A2/Flu conjugates (ranging from 0 to 1 µg/ml) and then tested for lysis by the relevant CTL at an E/T ratio of 10/1. As shown in Figure 4A, anti-CEA Fab-HLA-A2/Flu conjugate induced 50% lysis of LoVo cells at a concentration of 8 ng/ml, with no lysis of SK-BR-3 control cells, while anti-ErbB-2 Fab-HLA-A2/Flu conjugate induced 50% lysis of SK-BR-3 cells at 0.5 ng/ml, with a significant lysis of LoVo cells at higher concentrations (Fig. 4B). For anti-CD20 Fab-HLA-A2/Flu conjugate, 50% specific lysis of Daudi cells was induced at a concentration of 6 ng/ml. The tumor-associated antigen specificity was confirmed with no lysis of CD20 negative SK-BR-3 cells (Fig. 4C). Overall, the conjugates were active in the 10 to 100 picomolar range.

![Figure 4. Titration of the different bifunctional conjugates for induction of CTL mediated tumor cell lysis.](image)

Increasing concentrations ranging from 0 to 1 µg/ml (abscissa) of the three anti-tumor marker Fab-HLA-A2/Flu conjugates were incubated for 1 h at 37°C with different tumor target cell lines, followed by the addition of specific CTL for a 4 h 51Cr release assay. (A) Anti-CEA Fab-HLA-A2/Flu was incubated with LoVo cells (filled squares) or SK-BR-3 cells (open circles). (B) Anti-ErbB2 Fab-HLA-A2/Flu was incubated with SK-BR-3 cells (filled circles), LoVo cells (open squares) or Daudi cells (open diamonds). (C) Anti-CD20 Fab-HLA-A2/Flu was incubated with Daudi (filled diamonds) B lymphoma cells and with SK-BR-3 (open circles) carcinoma cells. Results are expressed as % specific target cell lysis. The conjugate concentration which induces 50% specific lysis is indicated in each panel.

There was only one instance of apparently non-specific CTL-mediated lysis, namely that induced by anti-ErbB-2 Fab-HLA-A2/Flu conjugate on LoVo cells (Fig. 3B and Fig. 4B), which express barely detectable amounts of ErbB-2 on cytofluorometry analysis (data not shown). However, competition experiments using unconjugated anti-ErbB-2 mAb indicated that CTL-mediated lysis was indeed specific. As shown in Figure 5, unconjugated anti-ErbB-2 mAb inhibited CTL-mediated lysis of LoVo cells to the same degree as that of SK-BR-3 cells. Interestingly, despite the presence of 20 µg/ml of competing anti-ErbB-2 mAb, anti-ErbB-2 Fab-HLA-A2/Flu conjugates were still able to induce maximal SK-BR-3 tumor cell lysis at concentration >0.1 µg/ml (Fig. 5), confirming the high potency of the conjugate.
Figure 5. Specific inhibition of the Fab-HLA-A2/Flu induction of CTL mediated tumor cell lysis by a competing antibody. To further demonstrate the antibody specificity of the induction of cancer cell lysis by Fab-HLA-A2/Flu conjugates, intact anti-ErbB-2 mAb (Herceptin) was added at 20 µg/ml (open symbols) or not added (filled symbols) with increasing concentrations of anti-ErbB2 Fab-HLA-A2/Flu on SK-BR-3 cells (A) or LoVo cells (B). Results are expressed as % specific target cell lysis.

Specific intracellular Ca\(^{2+}\) mobilization in CTL incubated with tumor cells coated with Fab-HLA-A2/Flu peptide conjugates

Figure 6. Specific induction of intracellular Ca\(^{2+}\) mobilization in cytotoxic T lymphocytes incubated with SK-BR-3 breast carcinoma cells coated with anti-ErbB-2 Fab-HLA-A2/Flu conjugates. Ca\(^{2+}\) mobilization was analyzed by cytofluorometry on the same influenza matrix peptide-specific T cell clone preincubated for 2 min at 37°C with (A) uncoated SK-BR-3 cells, (B) soluble conjugate at 5 µg/ml, (C) SK-BR-3 cells precoated by incubation with anti-ErbB2 Fab-HLA-A2/Flu conjugate at 5 µg/ml, (D) anti-CD3 mAb at 5 µg/ml crosslinked with goat anti-mouse antiserum. Left panels show the dot plot results for the 250 s recorded time. Right histogram panels show the percent of positive cells during the whole recorded time.
To determine the T-lymphocyte activation ability of Fab-HLA-A2/Flu conjugates targeted to tumor cells, Ca\(^{2+}\) mobilization assays \((20)\) were performed in influenza peptide-specific CTL. As shown in Figure 6, a strong and sustained Ca\(^{2+}\) mobilization was induced in CTL incubated with anti-ErbB-2 Fab-HLA-A2/Flu conjugates coated on SK-BR-3 cells, as compared to CTL incubated with an excess of the same conjugate in soluble form or with uncoated SK-BR-3 cells. The overall level of mobilized Ca\(^{2+}\) observed after incubation with conjugate coated SK-BR-3 cells was comparable to that achieved by standard anti-CD3 crosslinking. Thus, oligomerization of Fab-HLA-A2/Flu conjugates as a result of binding to the tumor antigens expressed by individual cancer cells played an essential role in CTL activation.

**Discussion**

The probable mechanism by which the conjugates, between the monomeric HLA-A2/Flu complexes and anti-tumor-associated antigen Fab' fragments described here, can induce efficient lysis of tumor cells by Flu-MA peptide-specific CTLs is schematically outlined in Figure 7. The key feature is that the complexes can activate CTL only when oligomerized on the surface of tumor cells. Thus, taking the old analogy of antibodies used as guided missiles, here the missiles would be fired only when they reach their targets. Moreover, because the conjugates will not induce significant activation when in solution, the amount of conjugates injected can be large enough to target all accessible tumor cells.

![Figure 7](http://www.cancerimmunity.org/v1p2/010101.htm)
the injected dose which is localized in the tumor is very low (7). Furthermore, recent cancer therapy trials using unlabeled anti-tumor antibodies, such as anti-ErbB-2 (8) and anti-CD20 (9) are based on the injection of large amounts of antibodies, which are well tolerated, in order to reach the highest number of disseminated cancer cells.

Another strategy aimed at redirecting T lymphocyte cytotoxicity against cancer cells consists in the use of bispecific antibodies with one arm directed against a tumor-associated antigen and the other against a T cell receptor-associated protein, such as CD3 (24, 25) or an NK cell-activating receptor, such as CD16 (26). Such bispecific antibody-based strategies, however, have limitations, which may explain why this form of therapy has not yet provided successful clinical results. First, the bispecific antibodies can react with all circulating T lymphocytes or NK cells before reaching the tumor cells. Second, the bispecific antibodies, if they reach the tumor site, are merely bridging tumor cells with the corresponding lymphocytes without necessarily triggering effector cell activation. In contrast, the strategy described here, which consists of targeting selected CTL epitopes, which become functional upon oligomerization on the surface of tumor cells, has a greater chance to fully activate specific CTL. Indeed, the oligomerized MHC class I/peptide complexes will provide multiple binding sites for specific CTL, not only via the T cell receptor but also via the CD8 molecule, which has been shown to play an essential role in the interaction with MHC class I/peptide complexes (27, 28, 29).

The idea of taking advantage of antigens targeted by antibodies on tumor cells to render them foreign, and possibly induce their rejection, is not entirely new. For instance, it has been shown that patients injected with murine anti-tumor antibodies developed a T cell response against peptide derived from xenoantibodies (30). It was even suggested that this T cell response may have been responsible for the rare cases of tumor remission following murine mAb therapy. To confirm and extend this hypothesis, we have previously coupled an immunogenic peptide from tetanus toxoid to a mAb directed against a B cell lymphoma idiotype. We showed that this peptide could be targeted to the lymphoma cells, processed and re-expressed in the context of MHC class II, leading to Fas-mediated target cell lysis by peptide-specific CD4 T lymphocytes (31, 32). However, this foreign peptide targeting approach was limited to MHC class II-expressing tumor cells. More recently, it has been shown that recombinant MHC class I/peptide complexes bound to CR1 B cells and K562 leukemia cells, via an anti-beta2M antibody (itself covalently coupled to the cell surface) could lead to lysis of the coated cells by specific CTL (33). However, no synthesis of conjugate between anti-tumor antibodies and MHC as described here, nor any functional binding tests to unmodified tumor cells, were reported.

Possible limitations to the clinical use of the Fab-MHC/peptide conjugates should be mentioned. First, there is the possibility that the peptide may dissociate from the MHC complex during the 12 to 24 h necessary for tumor targeting. Our preliminary results, however, show that the HLA-A2/Flu peptide complexes used in our conjugates have a dissociation half life of more than 48 h at 37°C in human serum, in agreement with other reports (11). Furthermore, there are efficient approaches, such as amino acid substitution (34) or introduction of a partial retro-inverso sequence (35), for increasing the affinity of peptides for the corresponding MHC molecules without markedly modifying the antigenicity of the complexes. In addition, it has been shown that recombinant human MHC class I molecules loaded with peptides can be synthesized as single chain molecules by using a fusion gene encoding the peptide, the heavy chain and beta2M almost without loss of antigenicity (36). The feasibility of generating in bacteria single chain human MHC complexes containing different peptides has been recently demonstrated (37). Single chain MHC complexes could represent ideal molecules for tumor targeting after coupling, or fusion, to anti-tumor Fab', or single-chain Fv fragments, respectively. The second limitation of the tumor targeting strategy described here is the limited time period during which the conjugates may be active in vivo. Thus, it may be advantageous to apply this strategy in conjunction with active T cell immunotherapy.

In this regard, we think that there are two major clinical situations in which the tumor targeting of Fab-MHC/peptide conjugates could be potentially useful. The first example concerns patients undergoing active T cell immunotherapy who do not show tumor regression, despite induction of a measurable T cell response against the immunizing antigen. In this situation, injection of Fab-MHC/peptide conjugates containing highly antigenic viral peptides derived from common viruses, such as influenza, Epstein-Barr virus or cytomegalovirus (against which the patient has an active T cell memory which could be boosted by additional viral vaccination) should mobilize the anti-viral CTL to react against the tumor cells, as demonstrated here in vitro. This temporary local induction of CTL-mediated tumor cell lysis may trigger the recruitment and full activation of T lymphocytes directed against autologous tumor antigens, by cross-priming events via apoptotic tumor cells (38) and cytokine production.
The second clinical situation concerns patients with partial tumor regression. Resistance of tumor cells to immunotherapy may be due to the development of HLA-loss variants \(^{(39)}\) or to the presence of tumor variants with a low surface expression of antigenic MHC/peptide complexes. Here, repeated injections of anti-tumor Fab-MHC/peptide conjugates containing peptide sequences derived from autologous antigenic peptides against which the patient has reacted, such as MAGE-A3 \(^{(40)}\), Melan-A/MART I \(^{(41, 42)}\) or NY-ESO-1 \(^{(43)}\), may promote CTL-mediated lysis of the remaining tumor cells.

In conclusion, the advantage of the Fab-MHC/peptide strategy described here is that it allows binding of many MHC class I complexes, each containing the same selected antigenic peptide, on the surface of individual tumor cells. In contrast, autologous tumor specific peptides synthesized by tumor cells have to compete with a host of other peptides for transport to the endoplasmic reticulum and for binding MHC class I molecules. Overall, tumor targeting of MHC/peptide complexes containing selected viral or autologous antigenic peptides, using the numerous high affinity anti-tumor-associated antigen antibodies presently available \((7, 14, 17, 18, 44, 45)\), may play a useful role in the difficult battle between T lymphocytes and tumor cells.

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**Abbreviations**

beta2M, beta2-microglobulin; CEA, carcinoembryonic antigen; Flu-MA, influenza matrix

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**References**


Materials and methods

Human cell lines and T cell clones

The colon carcinoma LoVo (ATCC CCL-229), the breast carcinoma SK-BR-3 (ATCC HTB-30) as well as the two B cell lymphomas Daudi (ATCC CCL-213) and Raji (ATCC CCL-86) expressing CEA, ErbB-2 and CD20, respectively, were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 supplemented with 10% FCS. The Daudi cells, which express no MHC class I due to the beta2M deletion, and the three other cell lines, known to be HLA-A2 negative, were confirmed not to react with the HLA-A2 specific mAb BB7-1 (6) used in the FACS analysis. The cytotoxic T lymphocyte clone NM 55 specific for the HLA-A2-restricted influenza matrix (Flu-MA) immunodominant peptide 58-66 was derived from a healthy normal and malignant B lymphocytes (18). F(ab')2 fragments were prepared by pepsin digestion (Sigma Chemical Co., St. Louis, MO) at a 3:100 (wt/wt) ratio of pepsin/IgG and incubated at 37°C in 0.2 M acetate buffer pH 4.0 for 22 h for mAb 35A7, 15 h for RITUXIMAB, or 8 h for HERCEPTIN, followed by gel filtration on a
Superdex 200 column (Pharmacia, Uppsala, Sweden). Fab' fragments were obtained by reduction of the F(\(ab\))\(_2\) with 10 mM cysteamine (Fluka, Buchs, Switzerland) for 1 h at 37°C in Hepes/NaCl buffer pH 7.0, followed by separation on Sephadex G25-PD10 columns (Pharmacia) as described (47).

**Generation of an MHC-peptide mutant**

A free cysteine instead of glutamic acid at position 275 and a stop codon at position 279 of the C-terminal part of the alpha3 domain of the HLA-A*0201 heavy chain were introduced by using the Quickchange site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The pHN1 HLA-A2-BSP (12) expression plasmid was the template for the polymerase chain reaction. The mutations were confirmed by sequencing.

Synthesis of the modified HLA-A2 peptide complex was performed according to previously described methods (12, 13, 15). Briefly, HLA-A*0201 heavy chain and beta2M were produced as inclusion bodies in *Escherichia coli*. Heavy chain, beta2M, and the HLA-A*0201-restricted Flu-MA\(_{58-66}\) (GILGFVFTL) immunodominant peptide were refolded by dilution and purified on a Resource Q column (Pharmacia).

**Fab fragment-HLA-peptide conjugate synthesis**

The purified HLA-A2 peptide complexes were incubated with a 25 molar excess of bismaleimide polyethylene oxide (Pierce, Rockford, IL) for 2 h at room temperature in phosphate buffered saline pH 7.0 (PBS). Excess coupling reagent was eliminated by gel filtration on a Superdex 200 column (Pharmacia). The bismaleimide-derivatized 45 kDa HLA-A2 peptide complex was immediately coupled to a 1.5 molar excess of freshly prepared reduced Fab' fragments followed by an 18 h incubation at 4°C, after concentration of the two proteins to 10 mg/ml. The conjugates were purified by FPLC on Superdex 200 columns and analyzed under nonreducing and reducing conditions by 10% SDS-PAGE gel electrophoresis. Concentration was determined by the Bradford method (Bio-Rad, Hercules, CA).

**Flow cytometry analysis**

Cells from the LoVo, SK-BR-3 and Daudi lines were incubated with each different Fab-HLA-A2/Flu conjugate in 50 \(\mu\)l of PBS containing 2% BSA, at a concentration of 2 \(\mu\)g/ml for 1 h at room temperature under gentle agitation. After three washings, FITC-labeled anti-HLA-A2 mAb BB7-1 (6) was added to the cells for an additional 30 min incubation at 4°C. Cells were then washed twice in PBS-2% BSA and immediately analyzed on a FACSCalibur® (Becton Dickinson, San Jose, CA).

**Chromium-release assay**

Tumor target cells (LoVo, SK-BR-3, Daudi or Raji) were incubated for 45 min at 37°C, with different concentrations of each of the three Fab-HLA-A2/Flu conjugates. During the same incubation, the tumor cells were \(^{51}\text{Cr}\) labeled. After three washings with PBS-2% BSA, the target cells (1000 cells per well) were incubated for 4 h at 37°C with the Flu-MA\(_{58-66}\) specific CTL (NM 55 CTL clone) (46) at the indicated effector to target cell ratios in 200 \(\mu\)l of DMEM, 10% FCS in V-bottomed microwell plates. Chromium release was measured and the percentage specific lysis was calculated as: 100 x [(experimental - spontaneous release) / (total - spontaneous release)] (6). In several control experiments, \(^{51}\text{Cr}\)-labeled tumor target cells were preincubated without conjugate or with an irrelevant Fab-HLA-A2/Flu conjugate and tested with the same CTL clone.

**Calcium mobilization analysis**

To measure the intracellular mobilization of calcium (Ca\(^{2+}\)) Flu-MA peptide specific CTL were preloaded with Indo-1 AM (Sigma) as described and then mixed at a 1:2 ratio with SK-BR-3 tumor cells precoated or not with anti-ErbB-2 Fab-HLA-A2/Flu conjugates at 5 \(\mu\)g/ml. The mixed cells, following a 1 min 1000 rpm centrifugation, were incubated for 1 min at 37°C before being analyzed by cytofluorimetry on a FACStar cytofluorometer (Becton Dickinson, Mountain View, CA) with a gating on the forward light scattering corresponding to cell conjugates. The positive control consisted of CTL incubated with murine anti-CD3 mAb at 5 \(\mu\)g/ml, crosslinked with anti-murine Fc antiserum, while the negative control consisted of CTL incubated for the same time at 37°C in culture medium alone. An important comparative test was performed by incubating CTL for the same time in the presence of soluble anti-ErbB-2 Fab-HLA-A2/Flu conjugate at 5 \(\mu\)g/ml.
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