Redirected cellular cytotoxicity by infection of effector cells with a recombinant vaccinia virus encoding a tumor-specific monoclonal antibody

Stéphane Paul,¹ Nadine Bizouarne,¹ Karine Dott,¹ Ludovic Ruet,¹ Patrick Dufour,² R. Bruce Acres,¹ and Marie Paule Kieny¹

¹Transgene S.A.11, Strasbourg, France; and ²Service d'Onco-Hématologie, Hôpital de Hautepierre, Strasbourg, France.

Cytotoxicity is an important function of the immune system that results in the destruction of cellular targets by humoral and/or cellular mechanisms. We wanted to assess the possibility of targeting the lytic function of immune cells toward cancer cells, which express the gene coding for a known tumor antigen (Ag) (GA733-2/epithelial cell adhesion molecule), using a viral vector encoding a monoclonal antibody (mAb) specific for said tumor Ag (CO17-1A). To this end, we have constructed recombinant vaccinia viruses expressing the sequences corresponding to mAb CO17-1A, which recognizes a specific Ag (GA733-2) that is present on the surface of most gastrointestinal carcinomas. The recombinant vectors encoding either a secreted or membrane-anchored form of CO17-1A mAb were used to infect effector cells, which were subsequently assessed for their cytotoxic activity. The recombinant viruses were able to infect both granulocyte-macrophage colony-stimulating factor-activated human macrophages and Ag-stimulated murine cytotoxic T lymphocytes. Infected granulocyte-macrophage colony-stimulating factor-activated macrophages were found to be able to kill GA733-2-expressing tumor cells. Likewise, infected cytotoxic T lymphocytes, although conserving their original alloreactivity, gained the capability of killing GA733-2-expressing cancer cells. **Cancer Gene Therapy (2000) 7, 615–623**

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The failure of cytotoxic therapy to induce long-lasting remission in the majority of patients harboring solid tumors has prompted investigations into new treatment modalities, including antitumoral immunotherapy. Many clinical attempts to recruit the humoral immune system for passive antitumoral immunotherapy have not fulfilled expectations.^{1–3} In addition, although many monoclonal antibodies (mAbs) specific for tumor-associated antigens (TAA) have been described, their therapeutic potential has essentially been limited to the situation of blood-borne tumors.⁴ Moreover, it has been suggested that soluble antibodies (Abs) can mask tumor antigens (Ags) (blocking Abs).⁴ The mechanisms of destruction of the tumor cells using unconjugated mAbs are not completely understood, but may involve various cellular host defense systems that require activation.³ Ab-dependent cellular cytotoxicity (ADCC) is thought to be an important effector function in antitumoral immunity, and various cell subpopulations (natural killer (NK) cells, T lymphocytes, macrophages/monocytes, and neutrophils) can participate in the ADCC killing of tumor

cells.⁵ Nevertheless, the limited *in vivo* concentration of the potentially active Abs and the difficulty of penetration of solid tumors by ADCC effector cells may explain in part the failure of soluble Abs in mediating clear therapeutic benefits. In addition, the absence of tumor-reactive cytotoxic T lymphocytes (CTLs), or their inability to respond adequately, can contribute to the immune escape of tumors.²

Two main approaches have been used to combine the effector function of cytotoxic cells with the antitumoral specificity of Abs. One of them is based on the use of bifunctional Abs directed both to a TAA and to a membrane structure capable of activating cytotoxic cells (for example T-cell receptor for T cells, CD16 for NK cells, and CD64 for macrophages).^{6,7} However, the therapeutic use of bispecific Abs is limited by the inaccessibility of many solid tumors to Ab penetration, and by the fast off-rate of Abs from the membrane of the effector cell.^{4,8} A second strategy involves the construction of chimeric receptors containing V_L/V_H single-chain V domains from TAA-specific mAbs linked to the Fc receptor-associated γ -chain or the T-cell receptor CD3 ϵ molecule with the goal of redirecting cytotoxic effector cells toward cancer cells.^{9–17} In this approach, the proposed mechanisms of cell killing clearly rely on signal transduction. This mode of treatment, although effective in animal models, is complicated and cumbersome.

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Address correspondence and reprint requests to Dr. Stéphane Paul, Transgene S.A.11, Rue de Molsheim, Strasbourg 67082, France. E-mail address: paul@transgene.fr

Figure 1. Schematic representation of the recombinant VVs. tk locus = *thymidine kinase* gene locus; P 7.5 = 7.5K vaccinia promoter; TMR = transmembrane and intracytoplasmic domains of rabies glycoprotein.

some. Indeed, retroviruses only tolerate insertion of relatively small transgenes, are notably difficult to purify to high titers, are easily inactivated, and will only infect proliferating effector lymphocytes. In contrast, poxviruses are amenable to the insertion of large segments of DNA, are very stable, can be produced at large scale, and can infect quiescent cells such as macrophages.

In this report, we describe a novel system for the redirection of cytotoxic cells using Abs. By co-expressing the heavy and light chains of a tumor-specific mAb in a vaccinia virus (VV) vector, we have been able to redirect the cytotoxic activity of activated cytotoxic cells to target cells expressing the tumor Ag. Gastrointestinal carcinoma-associated Ag (GA733-2), which is present on the surface of human tumors such as colorectal, pancreatic, and breast carcinomas, is recognized by the mAb CO17-1A.^{18–20} This mAb has been demonstrated to mediate cytotoxicity *in vitro* and tumoricidal activity in experimental animal models.^{18,19} Nonetheless, the antitumoral efficacy of unconjugated CO17-1A mAb is low in solid human tumors.²¹ Moreover, because a high tumor cell surface occupancy by the mAb is of importance for direct cytotoxic action, repeated high doses of mAb would probably have to be given to patients.²¹ As ADCC, or more generally membrane Ab-dependent cytotoxicity, is a key mechanism in the antitumoral effector function, increasing the killing capacity of cytotoxic cells might be beneficial to the therapeutic efficacy of the Ab.

MATERIALS AND METHODS

Tumor cells

Murine P815 cells (DBA2 mouse mastocytoma (H2D^d)) transfected with the human GA733-2 cDNA were established as

described previously.^{22,23} Murine RMA cells (C57BL/6 mouse T-cell lymphoma; American Type Culture Collection, Manassas, Va; (H2D^b)) were used to stimulate murine CTLs.

VVs

The complete heavy and light chains of mAb CO17-1A were introduced into the genome of VV (Copenhagen strain).²⁴ For pTG1142, the cDNAs coding for the heavy and light chains were introduced into plasmid pTG186poly downstream of two copies of the 7.5K vaccinia promoter.²⁵ The expression blocks were placed in reverse orientations to avoid homologous recombination between the two copies of the promoter. For the construction of pTG2179, the cDNA coding for the heavy chain of mAb CO17-1A was cloned in phase upstream of the sequence coding for the transmembrane and intracytoplasmic domains of rabies glycoprotein.²⁶ This sequence allows the heavy chain to be retained on the cell plasma membrane. As for pTG1142, the cDNAs for the native light chain and modified heavy chain were introduced into plasmid pTG186poly downstream of 7.5K vaccinia promoters, in reverse orientations. *In vitro* homologous recombination in primary chicken fibroblasts of VV DNA with plasmids pTG1142 and pTG2179 resulted in the generation of, respectively, VVTG1142 (VV CO17-1A sec), which synthesizes a secreted version of mAb CO17-1A, and VVTG2179 (VV CO17-1A tm), which produces a membrane-anchored version of the same mAb. VVTG186,²⁷ a thymidine kinase-negative virus devoid of transgene, was used as a negative control (Fig 1).

The procedure used for the purification of viral stocks generated a viral suspension devoid of recombinant Ab (data not shown).

Abs and cytokines

Murine immunoglobulin G2a (IgG2a) mAb CO17-1A was obtained from ascites fluid as described elsewhere.^{5,18,19} The estimated concentration of Ab in ascites fluid was 10 mg/mL. Fluorescein isothiocyanate (FITC)-labeled F(ab')₂ IgG goat

anti-mouse Ig was purchased from Jackson ImmunoResearch Laboratories (West Grove, Penn) and used for fluorescence-activated cell sorter (FACS) detection of murine CO17.1A mAb expression. FITC-conjugated mouse anti-human CD14 was purchased from Serotec (Oxford, UK). FITC-conjugated rat anti-mouse CD4 (L3T4) and FITC-conjugated rat anti-mouse CD8a (Ly2) were purchased from PharMingen (San Diego, Calif). Recombinant human granulocyte-macrophage colony-stimulating-factor (GM-CSF) and recombinant human interleukin-2 (rhIL-2) were purchased from R&D Systems (Abingdon, UK).

Establishment and maintenance of cytotoxic cells

Human macrophages. Human monocytes were isolated by leukapheresis and elutriation. *In vitro* differentiation of human monocytes in mature and functionally competent macrophages was achieved by 7 days of culture in the presence of 50 U/mL of recombinant human GM-CSF as described elsewhere.²⁸ The phenotype of the infected cells was verified by cytofluorimetry using a mouse anti-human CD14 mAb.

Murine CTLs. A total of 4×10^6 murine splenocytes from BALB/c mice (H2D^d) were stimulated with 2×10^6 mitomycin C (50 μ g/mL; Sigma, St. Louis, Mo) -treated murine RMA cells (H2D^b) for 7 days. At day 7, rhIL-2 (15 international units/mL) was added to the culture for 3 days of expansion. The CTL activity of these cells against RMA cells was measured by a ³⁺EuTDA cytotoxicity assay (Wallac Oy, EG&G, Turku, Finland). The phenotype of the infected cells was verified by cytofluorimetry using rat anti-mouse CD4 and CD8 mAbs.

VV infection of cytotoxic cells

Flow cytometry analysis. For the measurement of Ab expression by flow cytometry, cytotoxic cells were infected overnight at a multiplicity of infection (MOI) of ≤ 10 . After infection, VV-infected cells were ultraviolet (UV)-inactivated in uncovered 35-mm diameter cell culture plates (medium depth of liquid: 1 mm) by a 10-minute exposure under a 365-nm wavelength UV lamp (Orsam, Strasbourg, France) at a distance of 4 cm; next, they were washed extensively in complete culture medium before FACS analysis.

Evaluation of the cytotoxic activity of redirected effector cells. Cells were exposed to recombinant VV at a MOI of ≤ 10 for 1 hour at 25°C in phosphate-buffered saline. Infected cells were then UV-inactivated (see above) and extensively washed in complete medium to eliminate free replicative viruses before adding the target cells. Viability of UV-irradiated cells was at least 95% as measured by trypan blue exclusion. CO17-1A expression after UV treatment was verified by FACS analysis. No decrease in the expression of recombinant IgG was observed after UV treatment (data not shown).

Cytotoxicity assays

The cytotoxic activity of murine infected CTLs was determined by measurement of P815-GA733-2 lysis using a ³⁺EuTDA cytotoxicity assay (Wallac Oy).

The cytotoxic activity of human infected macrophages was determined in an 18-hour ⁵¹Cr release assay as described elsewhere.²⁹ As a control, activated macrophages were preincubated with CO17-1A mAb (10 μ g/mL) or with an irrelevant isotype-matched mAb (10 μ g/mL) for 1 hour at 37°C. The percentage of specific lysis was calculated using the following formula: % specific lysis = [(release in sample - spontaneous release)/[maximum release - spontaneous release]] $\times$ 100.

RESULTS

Construction of the recombinant VVs

Two recombinant viruses were constructed, VVTG2179 and VVTG1142, coding for a membrane-anchored and a secreted form of CO17-1A mAb, respectively (Fig 1). The natural signal peptides of the heavy and light chains of CO17-1A mAb allow transport of the recombinant Ab through the endoplasmic reticulum and Golgi apparatus to the plasma membrane. Utilization of the transmembrane domain of rabies G glycoprotein fused to the C terminal of the Ab heavy chain (VVTG2179) results in tethering of the heavy-light complex to the cell surface. It should be noted that no known signal transduction activity has been associated with the rabies glycoprotein. Ab CO17-1A made from VV-infected cells was correctly assembled as shown by the observation that IgG can be purified from supernatants of BHK-21 cells infected with VVTG-1142 using protein-A Sepharose. Using enzyme-linked immunosorbent assay quantification, we have determined that these supernatants contained ~ 1 μ g/mL IgG2a (data not shown), which is a typical figure for recombinant proteins produced from recombinant VVs.³⁰ Furthermore, the purified Ab was demonstrated by flow cytometry analysis to bind specifically to cells expressing Ag GA733-2 (P815-GA733-2) (data not shown).

Phenotypic characterization of the infected effector cells

Both recombinant viruses were evaluated for their capacity to infect effector cells such as GM-CSF-activated macrophages (Fig 2) or Ag-activated CTLs (Fig 3). The expression of CO17-1A mAb by macrophages on VV infection was assessed by flow cytometry analysis. Figure 2 shows a typical staining of the GM-CSF-stimulated adherent cell population, in which 70% of the cells were positive for CD14 expression before infection by the recombinant viruses. The efficiency of VV infection was assessed by measurement of CO17-1A expression. CO17-1A surface expression was weak (12%) for cells infected with VVTG1142, as expected for a secreted molecule. Infection with VVTG2179 allowed for CO17-1A expression on 35% of cells. Figure 3 shows a typical pattern of CD4 and CD8 expression by the allogeneic CTL population, in which 38% and 61% of cells were positive for CD4 and CD8 expression, respectively. On virus infection of these Ag-activated CTLs, $\sim 28\%$ of cells were found to express CO17-1A mAb independently of the recombinant virus used. While repeating this experiment, we have observed that higher MOIs result in increased CO17-1A expression (data not shown). The same experiments were performed with human NK or human lymphokine-activated killer cells without evidence of infection by VV (data not shown).

Cytotoxic activity of the infected effector cells

We subsequently examined the capacity of modified GM-CSF-differentiated monocytes to kill GA733-2-ex-

Figure 2. Flow cytometry analysis of CO17-1A mAb expression in GM-CSF-activated macrophages after VV infection. **A:** Determination of the cell phenotype before infection using a mouse mAb anti-human CD14. **B–D:** Determination of the expression of CO17-1A mAb after VV infection of GM-CSF-activated macrophages using FITC-labeled F(ab')₂ IgG goat anti-mouse Ig. **B:** VVTG186; **C:** VVTG1142; **D:** VVTG2179.

pressing murine tumor cells (P815-GA733-2) *in vitro* in a cytotoxicity assay (Fig 4). We first observed that significant background lysis (30%) occurred when the effector cells were infected with a negative control VV (VVTG186) (data not shown). We reasoned that this might be due to the destruction of labeled target cells after the release of infectious lytic virus from infected cytotoxic effector cells. We subsequently found that UV inactivation and extensive washing of infected macrophages were essential to reduce background lysis by VVTG186-infected effector cells. Nevertheless, this inactivation procedure also decreased the intensity of the positive signal (Fig 4). Indeed, preinfection of GM-CSF-activated macrophages with VVTG2179 followed by UV treatment resulted in 40% specific lysis of GA733-2-expressing tumor cells versus 85% without inactivation. As a positive control, cytotoxic cells preincubated with a high concentration of CO17-1A mAb were shown to be able to induce 40% specific lysis. Cells infected with VVTG186 (control VV), infected with VVTG1142, or loaded with an irrelevant isotype-matched mAb were not able to kill target cells.

Murine CTLs were obtained after a 1-week stimulation culture of BALB/c (H2D^d) spleen cells with allogeneic RMA cells (H2D^b) followed by 3 days of culture with rhuIL-2. Infection of these murine alloreactive

CTLs with either of the two CO17-1A recombinant VVs allowed redirection of the cytotoxic activity toward the GA733-2 Ag. Indeed, 38% and 40% specific lysis was observed in a ³⁺EuTDA release cytotoxicity assay with the viruses coding for the secreted (VVTG1142) and membrane-anchored (VVTG2179) CO17-1A mAb, respectively (Fig 5A). Cells infected with control VV (VVTG186) were not able to lyse GA733-2-expressing murine tumor cells. To investigate whether the infected murine CTLs still possessed their original Ag-specific cytotoxic activity, we assessed their capacity to efficiently kill RMA target cells in a parallel cytotoxicity assay (Fig 5B). This experiment showed that the CTLs maintained their anti-allogeneic specificity in addition to their newly acquired, mAb-directed specificity. Finally, to ensure that the redirected CTLs were indeed specifically recognizing GA733-2, their lytic activity was assessed on nontransfected P815 cells. No significant lysis of the P815 cells was observed independently of the recombinant virus tested (Fig 5C).

DISCUSSION

In this report, we have demonstrated that the nonspecific *in vitro* cytotoxic activity of human activated mac-

Figure 3. Flow cytometry analysis of CO17-1A mAb expression in alloreactive murine CTLs after VV infection. **A,B:** Determination of the cell phenotype using rat anti-mouse CD4 and CD8 mAbs. **C–E:** Determination of CO17-1A mAb expression after VV infection of RMA-stimulated CTLs using FITC-labeled F(ab')₂ IgG goat anti-mouse Ig. **C:** VVTG186; **D:** VVTG1142; **E:** VVTG2179.

Figure 4. mAb CO17-1A-mediated ADCC of P815-GA733-2 cells by GM-CSF-activated macrophages infected with VVs encoding for the secreted (**▲**, VVTG1142) or membrane-anchored (**○**, VVTG2179) CO17-1A mAb. The results, which are expressed as a percentage of specific ⁵¹Cr release, are the means of three separate experiments, with data points measured in triplicate. The SD was never >5% of the reported values (data not shown). As a positive control, CO17-1A mAb (10 μg/mL) preincubated macrophages were used. VVTG186 (**■**) infected macrophages and irrelevant isotype-matched mAb preincubated macrophages (Cont Ab, **×**) were used as negative controls.

Figure 5. mAb CO17-1A-mediated ADCC by Ag-activated murine CTLs infected with VVs coding for secreted ($\blacktriangle$, VVTG1142) or membrane-anchored ($\circ$, VVTG2179) CO17-1A mAb. **A:** P815-GA733-2 target cells; **B:** RMA target cells; **C:** P815 target cells. The results, which are expressed as a percentage of specific ^{51}Cr release, are the means of two separate experiments done in triplicate. The SD was never $>5\%$ of the reported values. Alloreactive murine CTLs infected with VVTG186⁶³ were used as negative controls.

rophages can be rendered tumor-specific upon infection with a recombinant VV that codes for the heavy and light chains of a tumor-specific mAb. One of the VV constructs used in these experiments encodes an Ab that is tethered to the cell membrane by the transmembrane peptide of the rabies virus glycoprotein. This is an important feature for several reasons: (a) because the rabies glycoprotein intracytoplasmic domain contains oligomerization sequences, it may increase the local concentration of mAb on the effector cell surface, which may facilitate contact with Ag-expressing tumor cells; (b) it ensures that the Ab stays anchored on the effector cells and does not passively mask the Ag present on the tumor cells. As uninfected human activated macrophages loaded with purified CO17-1A Ab were able to kill Ag-expressing tumor cells, it was surprising to observe that macrophages infected with the recombinant VV encoding the CO17-1A secreted form had no specific cytotoxic activity. Indeed, one might hypothesize that the secreted Ab would bind to the Fc receptor, but FACS analysis of the infected cells demonstrated that only marginal quantities of CO17-1A were present on the cell surface of the macrophages. In addition, we have observed that VV infection of human macrophages resulted in the loss of CD16 (human Fc receptor III) and CD14 (lipopolysaccharide receptor) membrane expression (data not shown). VV is known to inhibit the synthesis of cellular protein, and this could indeed explain the loss of CD14 and CD16 expression on infected macrophages and the inability of such cells to capture CO17-1A and to mediate ADCC using this pathway.³¹ Moreover, the fact that infected effector cells harboring the membrane-associated form of the Ab can kill target cells in the absence of soluble mAb suggests that the effector function might be independent of signaling through Fc receptors.

Another cytotoxic effector cell population, alloreactive CTLs, was also shown to be infectable with VV. FACS analysis of infected murine CTLs revealed that the patterns of membrane staining corresponding to the secreted and membrane-anchored forms of CO17-1A were virtually identical. These unexpected results suggest that "secreted" CO17-1A did in fact remain tethered on the cell membrane of murine CTLs. As a control, murine CTLs were infected with recombinant VVs coding for secreted or membrane-anchored versions of the gp120 env glycoprotein of HIV-1.³² The same results as for CO17-1A were obtained (i.e., both forms of env remained attached to the surface of CTLs). In contrast, only the membrane-anchored form could be found on the surface of activated macrophages or of BHK-21 cells (data not shown). These observations strongly suggest that the secretion potential of murine allogeneic CTLs or activated human macrophages for recombinant glycoproteins (CO17-1A and gp120) might be different, with secretion by the latter cell type being more efficient. The observation that both forms of CO17-1A mAb are present in equivalent quantities on the surface of infected CTLs is consistent with the fact that equivalent lysis activity is exerted by CTLs infected

with either of the two recombinant viruses. In addition, infected cells were shown to maintain their alloreactive CTL activity despite the acquisition of a new specificity for GA733-2-expressing cells. Because both CD4 and CD8 cytotoxic T cells were found in the allogeneic CTL population, it could not be determined which of these T cells were responsible for the killing of tumor cells.

Taken together, our results demonstrate that infected effector cells expressing the CO17-1A mAb on their surface are able to kill Ag-expressing tumor cells by a mechanism that we have called membrane Ab-dependent cytotoxicity. Lysis could be induced by specific activation of effector cells resulting from mAb/Ag complex formation or by a lectin-like behavior as suggested elsewhere.³³⁻³⁵ In the case of macrophages, a direct ADCC mechanism is unlikely to be involved. Indeed, the cytoplasmic portion of the recombinant modified heavy chain is devoid of a signal activation sequence. Thus, we surmise that the function of the hybrid Ab is to promote a cell-to-cell contact-dependent cytotoxic activity as described elsewhere.³⁶ In the case of CTLs, the same mechanism can be evoked, although it is also possible that the Ab associates with and signals through the CD3 complex, resulting in target cell killing.

GA733-2/epithelial cell adhesion molecule has been proposed to be an adhesion molecule that mediates calcium-independent homophilic interactions.³⁷ The possibility therefore exists that GA733-2 mediates interactions between the tumor cells used in this study and subpopulations of T cells.³⁸ Nevertheless, this phenomenon is unlikely to be responsible for the cytotoxic activity reported in this study, as effector cells infected with control VV are unable to kill target cells expressing GA733-2.

Ex vivo cytokine treatment of the mononuclear cells of cancer patients is a well-established technology for stimulating effector cells.²⁸ Therefore, the infection of cytokine-stimulated cytotoxic effector cells with a viral vector encoding a tumor-specific mAb is a realistic approach for the generation of tumor Ag-specific cytotoxic cells for cancer therapy. This approach combines the high specificity of TAA mAb with the cytotoxic activity and the tumor accumulation (homing of these effector cells). As we have observed that infected effector cells are less potent for the killing of tumor cells after UV treatment, it might be of interest to evaluate various protocols of virus inactivation, such as combinations of psoralen and long-wave UV light, which have been demonstrated to be less detrimental for cell viability than UV treatment.³⁹ In addition, optimal infection conditions should be determined to obtain a higher CO17-1A expression and therefore a potentially improved cytotoxic activity. Of importance is the fact that the technology allowing construction and large-scale production of recombinant VV is well established.^{25,27,30} More recently, it has been shown that highly attenuated modified vaccinia Ankara can be similarly manipulated, and this virus holds promise for an aggressive clinical use of recombinant VVs without the need for previous inactivation of infected effectors.⁴⁰

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