# Targeting of Cytotoxic T Cells Against Leukemic B Cells by Bispecific Antibody (aCD3 × aCD19) Does not Distract the T Cell From Its Primary Target[1]

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**Bispecific Abs (BsAb) represent a novel format of immunotherapy, recognizing immune effector cells (e.g., T cells), on the one hand, and target cells (e.g., tumor cells), on the other hand. To be successful, cross-linking of the two cell types is necessary for effector cell activation and subsequent killing of the malignant target cells. We asked the question, whether CTL that were incubated with the BsAb aCD3 × aCD19 and malignant B cells and activated to kill the malignant B cells were still able to eliminate their natural target cells (e.g., virus-infected autologous body cells). To test this, HLA-A*0201-restricted, influenza-specific CTL were incubated with BsAb- and HLA-A*0201-positive B lymphoid tumor cells in combination with HLA-A*0201-positive, virus-infected, non-B lymphoid cells as natural target cells. The results showed that even in the presence of BsAb and high amounts of tumor B cells, CTL were still capable of eliminating the virus-infected non-B lymphoid target cells; actually, CTL recognized and eliminated the homologous original target cells preferentially.** *The Journal of Immunology,* **1997, 159: 5545–5549.**

Bispecific Abs (BsAb)[3] consist of two different heavy and light chains and may bind to two different Ag present on different cell types. With their dual specificity, BsAb may recognize immune effector cells (e.g., T cells), on the one hand, and tumor target (e.g., malignant B) cells, on the other hand. To be successful, cross-linking of the two cell types is necessary (in combination with a second signal) for optimal effector (e.g., T cell) activation and subsequent cytotoxicity against the malignant (B) cells (1–3). The model BsAb aCD3 × aCD19 that we used can retarget autologous T cells to malignant B cells (4) and has been tested in non-Hodgkin's lymphoma (NHL). NHL is a frequently occurring malignancy of the lymphoid system in which the malignant cells in most cases belong to the B cell lineage and express the CD19 Ag.

In vitro studies have shown that B cells can be lysed by cytotoxic T cells in the presence of BsAb (4–7). The BsAb-mediated lysis was highly specific and independent of HLA class I expression (4, 8). A phase I trial using the BsAb CD3xCD19 showed little toxicity and some T cell activation (9). In vitro experiments suggested that the clinical responses to BsAb therapy could be enhanced by additional T cell activation, e.g., using a second signal such as IL-2 (10). In the following phase I/II trial, patients were treated with BsAb in combination with s.c. low dose IL-2 (11).

The study described here was initiated because of the first patient in that phase I/II trial. This patient (a 63-yr-old man) was diagnosed with chronic lymphocytic leukemia in 1989. He was refractory for Leukeran (Wellcome, England) and was treated with cyclophosphamide/vincristine/procarbazin. From December 1990 to March 1992, he was treated with fludarabine, which gave a partial response. After 1 yr, he relapsed and was treated again with fludarabine. In 1994, the chronic lymphocytic leukemia progressed, and the patient was treated six times with cyclophosphamide/adriamycin/vincristine/procarbazin without obvious results. In November 1994, this patient entered the phase I/II study, and he was treated with BsAb aCD3 × aCD19 in combination with s.c. IL-2. Some weeks later, the patient developed a severe herpes zoster (shingles pox) infection, which was treated with Aciclovir (Zovirax, Wellcome, England). We wondered whether this virus infection could be due to the treatment with BsAb or, as a more general question, whether T cells exposed to BsAb to kill malignant B cells would still be capable to eliminate their original target cells.

To address this question, we compared the effects of BsAb on the cytotoxicity of influenza-specific CTL for a virus-infected non-B cell line, SW620, as well as against the B lymphoid cell line JY (lymphoblastoid B cell) and vice versa. The results show that the process of virus-specific T cell cytotoxic killing prevails over BsAb-dependent B cell killing.

## Materials and Methods

### *Cell lines*

JY ($CD19^+$) is an EBV-transformed lymphoblastoid cell line and is positive for MHC class I HLA-A*0201 (12). JY was grown in RPMI 1640 Dutch Modification (Life Technologies, Paisley, Scotland) supplemented with 10% FCS and 20 μM 2-ME (Merck, Darmstadt, Germany).

SW620 is a colon adenocarcinoma (American Type Culture Collection CCL227, Rockville, MD) adherent cell line also expressing the MHC class

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[3] Abbreviations used in this paper: BsAb, bispecific antibodies; NHL, non-Hodgkin's lymphoma; L-15–10, Leibovitz L-15 medium supplemented with 10% fetal calf serum; ICAM-1, intercellular adhesion molecule-1.



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I molecule HLA-A*0201. Cells were grown in Leibovitz L-15 medium (Life Technologies) supplemented with 10% FCS (L-15–10; Life Technologies) and passaged weekly.

The SW620/CD19 cell line was developed by transfecting SW620 cells with vector pHZM-B7-19 carrying the cDNA encoding the human CD19 Ag (2). Cells were transfected by electroporation and selected in 250 and 500 μg/ml hygromycin (Calbiochem, La Jolla, CA). CD19 expression was tested by FACS analysis.

The influenza matrix-specific, HLA-A*0201-restricted CTL clone Q66.9 (a gift from Dr. H. Spits, Netherlands Cancer Institute, Amsterdam, The Netherlands) recognizes the influenza virus A/HK/68-encoded epitope GILGFVFTL. The CTL clone was grown on irradiated (30 Gy) HLA-A*0201-positive EBV-transformed B cells in RPMI 1640 supplemented with 10% human serum, 120 IU of rIL-2/ml (Eurocetus, Amsterdam, The Netherlands), and 1.5 μg/ml leukoagglutinin (Sigma Chemical Co., St. Louis, MO). Cells were tested for their cytotoxic activity, frozen in batches of $2 \times 10^6$ cells/vial, and kept in liquid nitrogen until use.

### *Infection with influenza virus*

SW620 cells were cultured for 48 h in the presence of 200 IU of human IFN-γ/ml (PeproTECH, Rocky Hill, NJ) before infection. Three to five million cells were infected with 100 infectious units of influenza virus A/HK/68 (Hong Kong 1968; a gift from Prof. Dr. Mazurel, Erasmus University, Rotterdam, The Netherlands). Cells were washed with L-15 containing 1% FCS, then incubated with virus for 1 h at 37°C. Cells were washed with L-15–10 and then cultured in L-15–10 for 16 h at 37°C.

### *BsAb CD3xCD19: SHR1*

The aCD3 × aCD19 BsAb-secreting clone SHR1 is a fusion product between the cell lines YTH12.5 and MG1CD19. YTH12.5 is a rat IgG2b mAb with a specificity for the human CD3ε Ag (13). MG1CD19 is a mouse IgG1 mAb and recognizes the human CD19 Ag. The production and purification of the BsAb SHR1 (aCD3 × aCD19) have been previously described (14). The BsAb SHR1 was used as complete Ab, including the Fc part.

### *$^{51}$Cr release assay*

Cytotoxicity was measured in a standard $^{51}$Cr release assay. All determinations were performed in triplicate. Briefly, $2 \times 10^6$ target cells were incubated with 100 μCi (3.7 MBq) of $Na_2\,^{51}CrO_4$ (Amersham, Aylesbury, U.K.) for 1 h at 37°C. After labeling, target cells were washed twice with medium and adjusted to $4 \times 10^4$ cell/ml, and 50 μl was added to 50 μl of various amounts of effector cells, 50 μl of BsAb/medium, and 50 μl of nonlabeled target cells, all seeded in wells of U-bottom microtiter plates (Costar, Badhoevedorp, The Netherlands) in a final volume of 200 μl. After 4 h of incubation the supernatant was harvested using the Skatron harvesting system (Skatron, Oslo, Norway), and chromium release was measured in a gamma counter (Minimax, Auto gamma counter, Packard Instrument Co, Meriden, CT). Maximum release was determined by incubating the target cells in 1% Triton X-100; spontaneous release was measured by incubating the target cells with medium alone. The percent specific $^{51}$Cr release was calculated using the formula: % specific release = [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100.

### *Cold target inhibition*

Possible inhibition of lysis of infected SW620 target cells was tested by preincubating Q66.9 effector cells with different amounts of unlabeled JY cells in the presence of BsAb. After 1 h of preincubation of JY and Q66.9 cells in the presence of BsAb at 37°C, the chromium-labeled SW620 target cells were added. Supernatant was harvested after 4 h of incubation.

Inhibition of BsAb-mediated lysis of JY cells was tested by simultaneously adding different amounts of unlabeled infected SW 620 target cells.

### *Immunofluorescence studies*

mAbs against CD3, CD19, CD20, CD45/CD14, HLA class I, and HLA-DR were purchased from Becton Dickinson (Becton Dickinson, Mountain View, CA). Abs binding to B7-1 (B7-24) and B7-2 (IG10, both unlabeled) were gifts from Dr. Mark de Boer (Innogenetics Ghent, Belgium). Abs binding to LFA-1 and ICAM-1 were gifts from Dr. Andries Bloem (Utrecht, The Netherlands). Unlabeled Abs were used in indirect stainings followed by goat anti-mouse Ig coupled to FITC. All mAb incubations and washing steps were performed at 4°C in PBS supplemented with 1% BSA and 0.01% $NaN_3$. Fluorescence was quantified using a FACScan (Becton Dickinson).

## Results

### *Killing of homologous target cells (SW620)*

The colon carcinoma cell line SW620 was either noninfected or infected with the influenza virus A/HK/68 and used as the target cell. The labeled cells were incubated with the cytotoxic T cell line Q66.9 in the absence or the presence of the B cell line JY and in the absence or the presence of the BsAb (Fig. 1*A* and Table I).

Cytotoxic T cells (Q66.9) did not kill noninfected SW620 target cells (negative control). Virus A/HK/68-infected target cells were properly killed (positive control), which was not affected by the presence of the BsAb. If B cells (JY) were added to the CTL plus virus-infected SW620 (SW620/V) target cells, the killing of the homologous target cells was not affected. The killing of virus-infected SW620 target cells by cytotoxic T cells in the presence of both B cells plus the BsAb was again not affected even with a 10- or 50-fold excess of the B cells. Collectively, the virus-infected target cells were killed in the same percentage range as those without the B cells and/or the BsAb.

### *BsAb-mediated killing of B cells (JY)*

The B cell line JY was labeled with $^{51}$Cr and incubated with the cytotoxic T cell clone Q66.9 in the presence or the absence of BsAb and in the presence or the absence of the homologous virus-infected non-B target cells (Fig. 1*B* and Table II).

Cytotoxic T cells (Q66.9) incubated with the B cell line JY but without the BsAb did not kill the B cells (negative control), as expected. In the presence of the BsAb the B cells were killed (positive control); this was not inhibited by the presence of non-infected SW620 target cells. T cell-dependent BsAb-mediated B cell kill was influenced, however, by the presence of virus-infected SW620 target cells; B cells were still killed but the presence of a minor proportion of virus-infected SW620 cells (0.1*SW620 = 10% of the total target cells) inhibited BsAb-mediated killing of the B cells. When virus-infected SW620 cells were added at a ratio of 1:1 (infected SW620 cells to B cells) or 10:1, the killing of the B cells was even more inhibited.

In conclusion, in the presence of BsAb and high amounts of tumor B cells, T cells still recognized the homologous target cell specificity and were able to eliminate these target cells preferentially. Actually, T cells preferentially killed the virus-infected non-B target cells. Even in the presence of high amounts of tumor B cells and BsAb, the original target cell-reactive killing of the specific T cells is maintained.

### *Killing of SW620/CD19 cells*

We tested whether differences in cytotoxicity toward virus-infected SW620 cells and the JY B cell line, respectively, as described above, were dependent on differences in the intrinsic susceptibility of the respective target cells. SW620 is an adherent growing carcinoma cell line, whereas JY is a nonadherent growing leukemic line. To approach this question, we transfected cell line SW620 with vector pHZM-B7-19, which carried cDNA encoding the human CD19 Ag and selected transfected cells with hygromycin. Together Figure 1 and 2 show that the cytotoxic T cells Q66.9 killed neither the original SW620 cells (negative control) (Fig 1) nor the CD19-expressing SW620 cells (SW620/CD19) (Fig. 2). These SW620/CD19 cells, however, were killed in the presence of BsAb (SW620/CD19 plus BsAb), but not to the same extent as the virus-infected SW620 cells (SW620/V, positive control).

## Discussion

Since a patient treated with BsAb and s.c. IL-2 developed a serious herpesvirus infection after the treatment, we wondered whether T

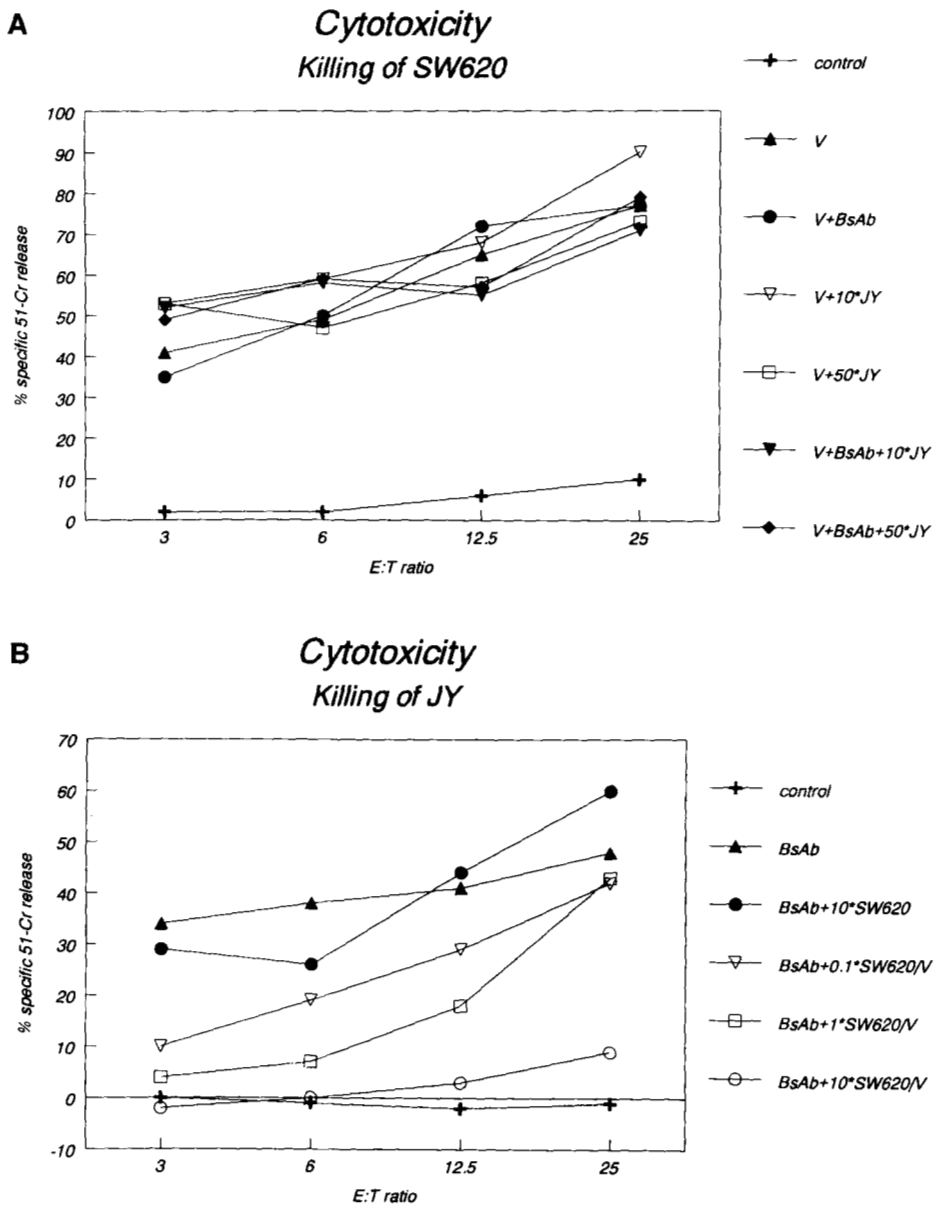


**FIGURE 1.** Cytotoxicity of T effector cells against natural target cells and B cells. The cytotoxicity of the effector cells Q66.9 against the target cells SW620 (colon carcinoma; *A*) or JY (B cell; *B*) is assayed in a $^{51}Cr$ release assay. Target cells SW620 or JY were incubated with the effector cells Q66.9 under the conditions indicated with or without the BsAb and with or without the other (cold) target cells as possible competitors. The percentage of $^{51}Cr$ release is displayed at various E:T cell ratios. The colon carcinoma cells were either noninfected (control) or infected with the influenza virus (V). Cold target cells were used in a 0.1-, 1-, 10-, or 50-fold excess.

Table I. *Killing of infected target cells (SW620/AHK) by T effector cells*

| $^{51}Cr$ Labeled SW620 Target Cells | Lysis | |
|---|---|---|
| | Expected | Observed |
| Cytotoxic T cells + noninfected target cells | –[a] | – |
| Cytotoxic T cells + infected target cells | +[b] | + |
| Cytotoxic T cells + infected target cells + B cells (JY) 10x/50x[c] | + | + |
| Cytotoxic T cells + infected target cells + BsAb | + | + |
| Cytotoxic T cells + infected target cells + B cells (JY)10x + BsAb | ? | + |
| Cytotoxic T cells + infected target calls + B cells (JY)50x + BsAb | ? | + |

[a] –, no killing.
[b] +, killing.
[c] 10×/50× more B cells (JY) than infected target cells (SW620).

Table II. *Killing of B cells (JY) by T effector cells*

| $^{51}Cr$ Labeled JY B Cells | Lysis[a] | |
|---|---|---|
| | Expected | Observed |
| Cytotoxic T cells + B cells (JY) | – | – |
| Cytotoxic T cells + B cells (JY) + BsAB | + | + |
| Cytotoxic T cells + B cells (JY) + noninfected target cells + BsAb | + | + |
| Cytotoxic T cells + B cells (JY) + infected target cells (10x) + BsAb | ? | (–) |
| Cytotoxic T cells + B cells (JY) + infected target cells (1x) + BsAb | ? | (+) |
| Cytotoxic T cells + B cells (JY) + infected target cells (0.1x) + BsAb | ? | + |

[a] –, no killing; +, killing; (+), reduced killing; (–), highly reduced killing; ?, undetermined.

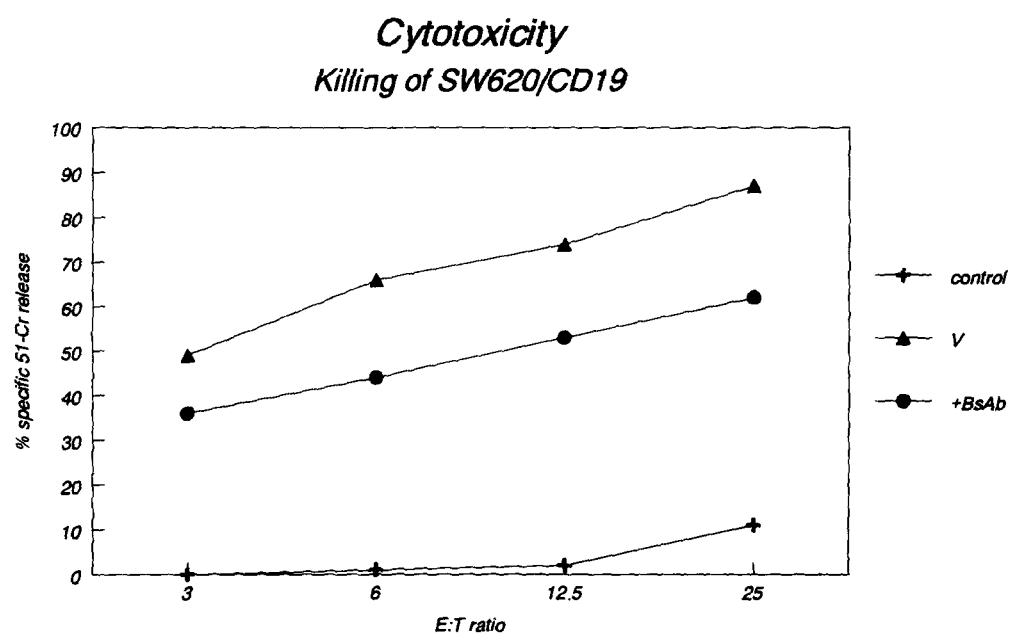


**FIGURE 2.** Cytotoxicity of T effector cells against CD19-transfected SW620 cells (SW620/CD19). The cytotoxicity of the effector cells Q66.9 against the CD19-transfected SW620 (colon carcinoma) (SW620/CD19) is analyzed in a $^{51}Cr$ release assay. Target cells SW620 or SW620/CD19 were incubated with the effector cells Q66.9 under the conditions indicated with or without the BsAb. The percentage of $^{51}Cr$ release is displayed at various E:T cell ratios. The colon carcinoma cells were either noninfected (control) or infected with the influenza virus (V).

cells that are targeted with BsAb toward malignant B cells were still able to defend the body against infectious agents and whether the treatment with BsAb would induce a T cell immunodeficiency. The experiments performed here to address this question showed that T cells were still capable of recognizing their natural target cells and kill them; in fact, the natural target cells were killed preferentially, even with preincubation of T cells and B cells in the presence of the BsAb and later addition of non-B natural target cells.

Why do the T cells prefer the natural virus-infected target cells SW620 in the presence of B cells JY and BsAb? Three points merit attention, i.e., the mechanisms governing the interaction between effector and target cells, the mechanism of cytotoxicity in the effector cell, and the comparative susceptibilities of the two target cells.

In the first point (the avidity of the interaction between effector and target cells), three aspects merit discussion, i.e., the affinity of the specific binding, the presence of accessory molecules, and the comparative antigen densities. The TCR complex recognizes a peptide lodged in the peptide binding groove of MHC class I or II molecules with an affinity of about $10^{-5}$ M (15, 16). The affinity of this specific interaction is lower than that in the BsAb system, as both CD3 and CD19 parental Ab expressed affinities in the range of $10^{-8}$ to $10^{-9}$ M. The affinities of the CD3 and CD19 parts of the BsAb were similar to those of the parental mAbs (17), indicating that the affinity of the specific BsAb-mediated T-B interaction supersedes that of the T-SW620 interaction.

To enhance the affinity of cellular interactions, additional ligands are involved in binding and signaling. These accessory molecules in the interaction between cytotoxic T cells and their target cells may include LFA-1, CD2, and CD28. ICAM-1 is known to interact with LFA-1, which is present on all immune cells. The CD2 molecule on T cells is also involved in T cell activation in conjunction with TCR; it is a receptor for LFA-3, which is expressed on many different cell types and present on all APCs. The most important costimulatory molecules known are termed B7-1 (CD80) and B7-2 (CD86), which are ligands for CD28 as well as its homologue CTLA-4, a molecule that is expressed on T cells and up-regulated during T cell activation. In these experiments, the HLA class I Ag expression on the B cells and that on the colon carcinoma cell line SW620 are identical, and HLA class I differences should not play any role. Moreover, it has been shown that BsAb-mediated killing is independent of HLA class I differences or expression of ICAM-1, B7, and LFA-1 molecules (4, 10). The target cells were tested for the expression of second signal molecules; JY cells are positive for B7-1, B7-2, and ICAM-1, whereas the virus-infected target cell line SW620 does not express these accessory molecules (Table III).

Ag densities governing specific interaction may pertain to CD3 (identical in both systems) and CD19 vs viral peptide-expressing MHC class I molecules. By using viral infection in culture (as used here), Falk et al. found that the proportion of viral peptide-loaded MHC molecules approximates 0.1 to 0.5% of the total expression of MHC class I molecules (18, 19); the latter is in the same range as the expression of CD19 on the B cells. All three aspects mentioned for the avidity of the interaction (i.e., affinity, accessory molecules, and Ag density) seem to favor the BsAb-mediated T-B cell interaction, which is apparently not the case.

The two other factors include mechanism of cytotoxicity (in both systems dependent of the granzyme/perforin pathway (S. C. Klein, unpublished observations) or susceptibility of the target cells. Reportedly, necessary E:T cell ratios in T cell-dependent, BsAb-mediated tumor killing are higher in carcinoma cell lines than in B cells (8), possibly indicating a lower intrinsic susceptibility of adherent growing carcinoma cells for T cell lytic processes. Our results might indicate just the reverse situation, i.e., higher sensitivity of the virus-infected carcinoma cells. To test whether the two different mechanisms of recognition taking place on the cell surface of the same cell might result in different levels of induced cytotoxicity, we transfected SW620 cells with CD19 cDNA and tested the susceptibility of these cells in a BsAb-mediated system compared with that in virus-infected SW620 cells.

Table III. *Expression of cell surface markers as determined by immunofluorescence*

| Cell Line | SW620 | JY |
|---|---|---|
| ICAM-1 | − | + |
| B7-1 | − | + |
| B7-2 | − | + |
| LFA1 | − | + |
| MHC class I | + | + |
| MHC class II | − | + |
| CD19 | − | + |
| CD20 | − | + |

As the density of CD19 on the SW620/CD19 cell line is comparable with that of JY, and as intrinsic differences in both target cell susceptibility and effector cell efficacy can be ruled out in this system, these findings reinforce the idea that virus-dependent killing prevails over BsAb-mediated killing. Given the preference for virus-specific killing over BsAb-mediated killing, as shown here, what, then, is the prospect of BsAb-mediated treatment of malignancies? As the frequency of T cells specific for a given Ag is low even in acute viral infections, a host of other T cells can be involved in BsAb-mediated B cell killing.

Collectively, despite the "less favored" conditions, low TCR affinity, no expression of accessory molecules, and low Ag density, the virus-infected target cells are preferred over BsAb-mediated killing; differences between processes of cytotoxicity or sensitivity of cells cannot explain this preference.

However, very recent data on T cell activation seem to indicate that a low avidity of the MHC class II/TCR interaction suffices, or may even be preferred, for T cell activation (20). To our knowledge, it is not known whether this preference also applies to the MHC class I/TCR interaction of cytotoxic T cells; our data on the comparison of three different anti-CD3/CD19 BsAb in the mediation of T cell cytotoxicity from preactivated T cells seem to indicate a positive influence of the affinity of CD3 recognition.

Apart from these basic immunologic aspects, the presented data deserve clinical interest as well. In iatrogenic T cell immunodeficiencies, e.g., after transplantation and consecutive immunosuppression, endogenous viruses, especially herpesviruses such as HSV, VZV, CMV, and EBV, can recur and give rise to problems (21). Also, de novo infections may not be dealt with properly (22). In the patient who motivated these studies, the recurrent HSV infection is unlikely to be due to the experimental BsAb treatment for his NHL, however. Alternatively, his pretreatment situation involving fludarabine probably underlies this recurrent infection.

Finally, these findings pertain to tumor immunology as well. In the murine system, possibly through enforced Ag presentation, BsAb treatment seems to result in a tumor-specific immunity. Mice bearing transplantable EBV-infected tumorigenic B cells could be treated successfully with BsAb on day 3 after transplantation. When these treated mice were rechallenged with the same tumor, they were resistant in tumor growth, apparently specifically, as another transplantable tumor was not rejected (23). Our findings indicate that this tumor-specific immunity will not be hampered by further addition of BsAb.

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