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Brefeldin A Blocks the Cytotoxicity of T Cell Receptor α/β and γ/δ Cytotoxic T Lymphocyte Clones Reacting against Human Autologous Cancer Cells

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We studied the effector mechanism of T cell receptor (TCR) α/β- and γ/δ-type cytotoxic T lymphocyte (CTL) clones that react with human autologous tumor cells. Treatment of tumor cells with a fungal antibacterial reagent, brefeldin A (BFA), resulted in the inhibition of cytotoxicity of an autologous tumor (HST-2)-specific $CD8^+$ TCR α/β-type CTL, TcHST-2. Other anti-metabolites such as chloroquine, cycloheximide and colchicine did not affect the cytotoxicity. The cell-surface antigen expression, including MHC class I molecules, was not influenced by BFA treatment. Furthermore, BFA did not influence the cytotoxicity of lymphokine-activated killer cells and natural killer cells. Since BFA blocks the transport of peptides from endoplasmic reticulum to the Golgi apparatus, the above data suggest that BFA could affect washing out of the peptide fragments from the MHC class I groove. Consequently, target tumor cells were protected from killing by CTL. Moreover, we obtained a $CD4^-$, 8^-, TCR γ/δ-type ($V\delta1^+$) CTL clone, TcHOT, that reacts against an autologous ovarial carcinoma, HOT. BFA could also inhibit this cytotoxicity, and it is likely that different presenting molecules other than MHC class I proteins participate in the cytotoxicity of this TCR γ/δ-type CTL. These studies suggest that both TCR α/β- and γ/δ-type CTL may require antigenic peptides that are most likely derived from the BFA-sensitive, intracellular endogenous target proteins.

Key words: Brefeldin A — CTL — Human autologous tumor

The mechanisms through which human autologous tumor cells are killed by CTL[4] are largely unknown. However, since recombinant cytokines such as rIL-2, rIL-4 and rIL-6 have become available for laboratory use, *in vitro* cultivation and the cloning of CTL have been greatly improved. There have been reports indicating the direct cytotoxicity of MHC class I-restricted CTL clones that react specifically against autologous tumor cells of various tissue origins.[1–6] These studies directly demonstrated that patients' T cells could specifically respond to their own tumor cells.

The usage of TCR V α/β genes indicated that certain tumor antigens are involved in the cytotoxicity of CTL to autologous tumor cells.[7] In most of the previous studies, MHC class I restriction of the CTL cytotoxicity was confirmed by blocking of the cytotoxicity using anti-MHC class I mAb.[1, 3, 8, 9] Until recently, it has been difficult to study the association of endogenous antigenic peptides with MHC class I molecules. Therefore, although the cytotoxicity was MHC class I-restricted, we could not ascertain whether the antigenic peptides participate in the cytotoxicity of CTL to tumor cells.

This problem may now be analyzed owing to the availability of a fatty acid analogue, BFA.[10–13] Nuchtern *et al.* clearly demonstrated that an influenza A-specific cytotoxicity of $CD8^+$ CTL was blocked by the treatment of target cells with BFA.[14] It has been demonstrated that BFA blocks the transport of cellular peptides from ER to the Golgi apparatus by fusing these two organellas.[15, 16] In other words, the association of antigenic peptides with MHC class I molecules is possibly inhibited in these compartments of the cells by BFA treatment. Consequently, the susceptibility of target cells to CTL is lost.[14]

In this paper, we first studied the blocking effect of BFA on the cytotoxicity of an autologous tumor (HST-2)-specific TCR α/β-type CTL clone, TcHST-2. Second, we also observed a similar effect of BFA on the cytotoxicity of a TCR γ/δ-type ($V\delta1^+$) CTL clone, TcHOT, reacting against an autologous ovarial carcinoma, HOT. We will discuss the nature of the antigenic peptides and presenting molecules in these human autologous tumor-CTL interactions.

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[4] Abbreviations: CTL, cytotoxic T lymphocytes; BFA, brefeldin A; MLTC, mixed lymphocyte-autologous tumor cell culture; LAK, lymphokine-activated killer; NK, natural killer; TCR, T cell receptor; rIL, recombinant interleukin; MHC, major histocompatibility complex; FACS, fluorescein-activated cell sorter; mAb, monoclonal antibody; ER endoplasmic reticulum; PCR, polymerase chain reaction; ADCC, antibody-dependent cellular cytotoxicity; PBL, peripheral blood lymphocytes.

MATERIALS AND METHODS

Establishment of CTL and human autologous tumor lines The procedure for establishment of pairs of CTL and autologous tumor lines was the same as described in our previous papers.[1, 17, 18] In this study, we used two pairs of CTL and human autologous tumor targets. HST-2 was a gastric signet ring cell carcinoma line. CTL reacting against HST-2 was established from PBL of this patient by MLTC.[19] Finally we isolated the $CD2^+$, 3^+, 4^-, 8^+, $11a^+$, 16^-, $45RO^+$, 56^-, TCR α/β^+ functional CTL clone, designated as TcHST-2 (HLA-A2, A31, B38, B54, C1, C7, DR4.1, DR4.2, DRw53, DQ3, DQ4). The cytotoxicity of TcHST-2 to HST-2 target was blocked by anti-CD8[19] and anti-MHC class I mAbs, indicating that TcHST-2 is a conventional MHC class I-restricted CTL. Second, a HOT line was derived from the ascitic fluid of a 58-year-old female patient with ovarial carcinoma, and $CD2^-$, 3^+, 4^-, 8^-, $11a^+$, 16^-, $45RO^+$, 56^-, TCR$\gamma 9^-$, $\delta 1^+$, $\delta 2^-$ CLT was obtained by MLTC three times. This T cell clone was designated as TcHOT (HLA-A26, B35, B60, C7, DR9, DR12, DRw52, DRw53, DQ3, DQ7). These CTL clones were usually stimulated with MLTC for 2 days. Then cells were maintained and proliferated for a further 5 days *in vitro* in the presence of 350 U/ml of rIL-2 (Shionogi Pharmaceutical Co., Tokyo). Two days before the cytotoxicity assays, rIL-2 was depleted from the culture, since this treatment confers specific reactivity to the autologous tumor targets of CTL.[19]

Cytotoxicity, allogeneic tumor lines, LAK and NK cells The cytotoxicity assay was previously described.[1, 18, 19] In the present study, in addition to autologous tumor cells, we used several allogeneic lines (Daudi, K562, U937) and mouse cell line P815. LAK cells were obtained by culturing allogeneic human peripheral T cells in the presence of 350 U/ml of rIL-2 for 7 days. We also used allogeneic NK cells from peripheral resting T cells in the cytotoxicity experiment, as previously described.[18]

mAbs, FACS analysis and treatment in the cytotoxicity assays In the analysis of cell surface phenotype of cells and in the cytotoxicity study, we used mAbs. Antibodies to CD3, 4 and 8 were purchased from Ortho Diagnostic Systems Co., Tokyo. Antibody to CD3 with IgM isotype (clone HB231) was obtained from American Type Culture Collection (ATCC). mAbs that detect specific V region determinants on TCR γ/δ-type T cells were also utilized. These mAbs were TiγA (a gift from Dr. T. Hercend),[20] BB3 and A13 (gifts from Dr. L. Moretta).[21] TiγA recognizes a determinant on Vγ9, and BB3 and A13 those on Vδ2 and Vδ1, respectively. Antibodies HH-1 and TC-8B1 that react with determinants on the human MHC class I and class II framework structures, respectively, were developed in our own laboratory.[18] Anti-MHC class I W6/32 mAb was purchased from ATCC. mAb (HA-58) reacting against ICAM-1 was obtained from Dainippon Pharmaceutical Co., Osaka. We also developed hybridomas which secrete mAbs (7D3, 1C1) reacting against non-MHC class I and class II cell surface antigens expressed on HST-2 cells. The hybridoma production was carried out in the same manner as described in our previous papers.[18, 22] The procedures for FACS analysis and the blocking study of the CTL cytotoxicity using these mAbs were described elsewhere.[22]

Treatment with BFA, chloroquine, cycloheximide and colchicine We assessed the effect of several antimetabolic reagents on the susceptibility of target tumor cells to CTL cytotoxicity. ^{51}Cr-labeled target cells were pretreated in U-bottomed microtiter plates with various concentrations of BFA (kindly provided by Drs. D. Romer and E. Rissi, Sandoz Pharma AZ, Switzerland) for 30 min at 37°C in a CO_2 incubator.[14] We usually employed 2.5 μg/ml of BFA. Target cells were also pretreated with 60 μM chloroquine (Sigma Chemical Co., St. Louis, Mo.) for 4 h at 37°C,[13] with 20 μM cycloheximide (Sigma Chemical Co.) for 4 h[23, 24] and with 500 μg/ml of colchicine (Sigma Chemical Co.) for 4 h.[25] The cytotoxic T cells were then added and culture was continued for another 10 h in the presence of the same amounts of these reagents. In some experiments, target cells were treated with BFA for 30 min as described above, but the cytotoxicity test cultures were done in the absence of BFA. In order to assess the influence of treatment with these reagents on the cell surface antigen expression, tumor cells were treated with BFA in a similar manner to that described above. Then a FACS study was done for the cell surface antigen expression by using appropriate mAbs.

RESULTS

Effect of BFA on the cytotoxicity of TCR α/β-type CTL to autologous tumor targets In order to study whether the antigenic peptides are involved in the cytotoxicity of TcHST-2 specifically reacting against autologous tumor HST-2,[19] we used BFA. We also employed other antimetabolic reagents such as chloroquine (blocks the transport between Golgi and endolysosomes), cycloheximide (blocks the elongation of peptides) and colchicine (disrupts organization of microtubules). ^{51}Cr-labeled HST-2 cells were pretreated with appropriate concentrations of these reagents, and the cytotoxicity assays with TcHST-2 were performed in the presence of these reagents. HST-2 target cells treated with BFA were clearly protected from the cytotoxicity of TcHST-2 by BFA in a dose-dependent manner (data not shown). As shown in Fig. 1a, 2.5 μg/ml of BFA gave strong inhibition of the cytotoxicity. However, treatment with chloroquine,

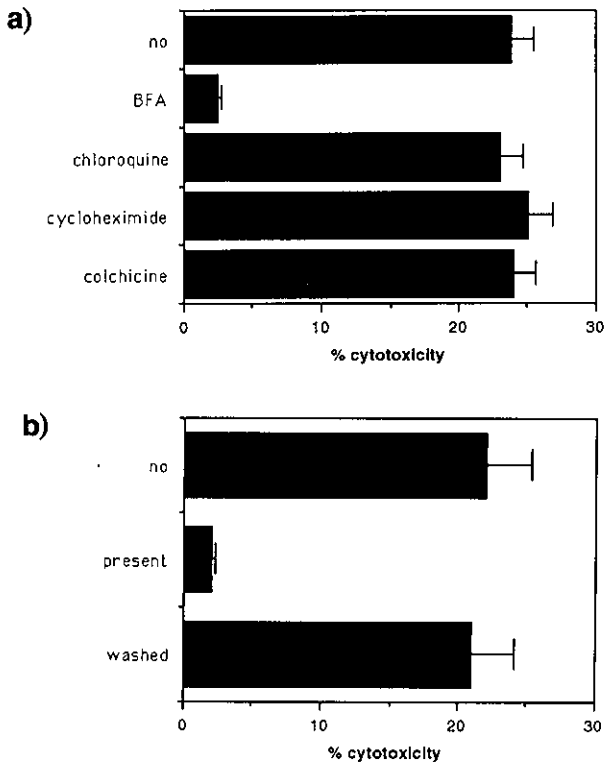

Fig. 1. a) Comparison of the effect of BFA on TcHST-2 cytotoxicity against HST-2 autologous tumor cell targets with that of other antimetabolites. ^{51}Cr-Labeled HST-2 cells were pretreated with BFA, chloroquine, cycloheximide and colchicine, as described in "Materials and Methods." TcHST-2 cells were then added at an E/T ratio of 25, and were cultured for 10 h in the presence of these reagents. b) The effect of BFA may be reversible. ^{51}Cr-Labeled HST-2 cells were pretreated with 2.5 μg/ml of BFA for 30 min at 37°C, and then washed with PBS. TcHST-2 cells were added, and cultured in the presence or absence (washed) of the same amount of BFA. Bars represent ±SE.

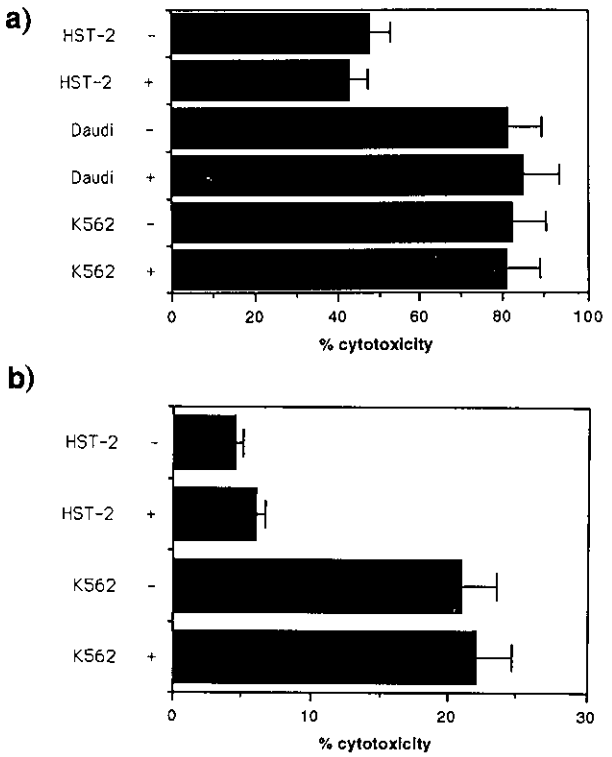

Fig. 2. Effect of BFA on the cytotoxicity of allogeneic LAK (a) and NK (b) cells against HST-2, Daudi and K562 target cells. LAK cells were obtained by culturing allogeneic peripheral T cells in the presence of 350 U/ml of rIL-2 for 7 days. NK cells were obtained from peripheral resting T cells. ^{51}Cr-Labeled target cells were pretreated with 2.5 μg/ml of BFA for 30 min at 37°C. Then, the cytotoxic cultures were done in the presence (+) of the same amount of BFA at an E/T ratio of 100 for 12 h. They were also done without BFA pretreatment and in the absence of BFA (−). Bars represent ±SE.

cycloheximide and colchicine did not affect the cytotoxicity. Furthermore, BFA was used to pretreat target cells for 30 min at 37°C, then washed out from the culture, and the cytotoxicity assay was done in the absence of BFA. The data showed that BFA-washed-out target cells became susceptible to lysis by CTL (Fig. 1b). This indicates that the effect of BFA on target tumor cells is reversible.

Because the presence of BFA in the cytotoxicity assays was required to inhibit the cytotoxicity, the possibility arises that the effect of BFA might occur at the effector T cell rather than at the target cell level. In order to clarify this, we assessed the effect of BFA on the cytotoxicity by reverse-ADCC of TcHST-2 against IgG-Fc receptor (+) U937 target cells. When TcHST-2 was treated with anti-CD3 mAb with IgG isotype, the cytotoxicity was clearly enhanced. However, BFA did not much affect this cytotoxicity; there was only a minimal inhibitory effect, indicating that the inhibitory effect of BFA on the cytotoxicity of TcHST-2 operates predominantly at the target cell level (data not shown).

We also examined whether BFA could affect the cytotoxicity of allogeneic LAK and NK cells against HST-2, Daudi and K562 target cells. Fig. 2a showed that the cytotoxicity of LAK against HST-2, Daudi and K562 cells was not influenced by BFA. This was also true for NK cytotoxicity to K562 targets. Peripheral NK cells were not cytotoxic to HST-2 (Fig. 2b). These data suggest that BFA does not affect the mechanisms of LAK and NK cytotoxicity.

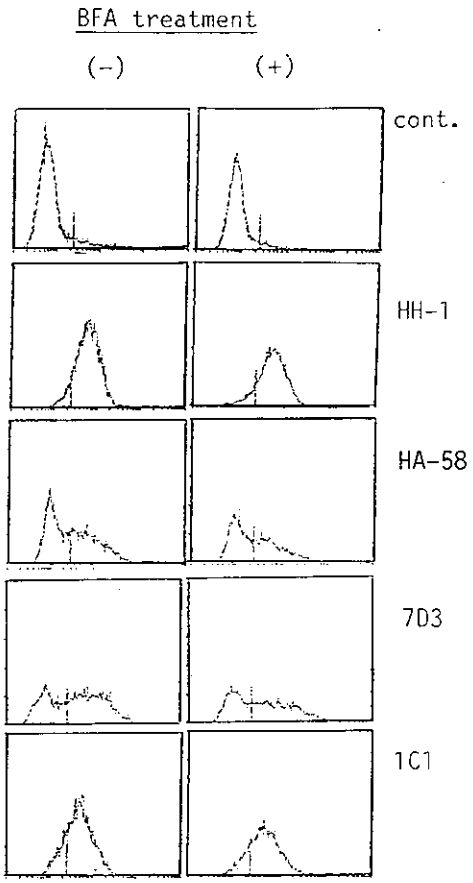

Fig. 3. Effect of BFA on the expression of certain cell surface antigens on HST-2 cells as assessed by mAbs. HST-2 cells were treated (+) with 2.5 μg/ml of BFA for 30 min at 37°C, and then were reacted with mAbs which detect MHC class I (HH-1), ICAM-1 (HA-58) and non-MHC class I and class II molecules (7D3, 1C1) for 60 min at 4°C. FITC-conjugated goat-anti mouse Ig was added, and FACS analysis was done. (−) indicates non-treated controls of HST-2 cells.

We also investigated the influence of BFA treatment of tumor cells on the cell surface antigen expression. In this study, mAbs reacting to MHC class I nonpolymorphic determinant (HH-1), ICAM-1 (HA-58) and non-MHC class I antigens (7D3 and 1C1) were employed. HST-2 cells were pretreated with 2.5 μg/ml of BFA for 30 min at 37°C, and allowed to react with mAbs in the presence of the same amount of BFA for 60 min at 4°C. Cells were washed with PBS, and allowed to react with FITC-conjugated goat anti-mouse Ig for 60 min at 4°C. As shown in Fig. 3, FACS analysis indicates that there was no obvious change of these antigen expressions. It should also be mentioned that the TcHST-2 antigen expression, such as CD2, 3, 8 and TCR α/β, was not influenced by BFA treatment (data not shown).

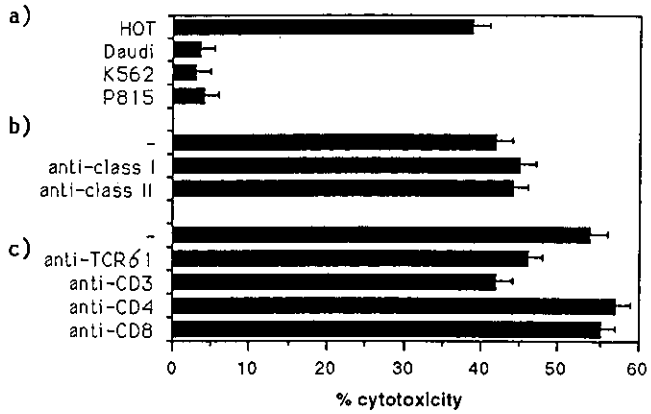

Fig. 4. Cytotoxicity profiles of TCRδ1$^+$ TcHOT. a) Cytotoxicity of TcHOT against an autologous target, HOT, and other cell lines. TcHOT and ^{51}Cr-labeled target cells were mixed at an E/T ratio of 25. b and c) The blocking of TcHOT cytotoxicity against HOT targets. In experiment b, ^{51}Cr-labeled HOT cells were treated with a saturating amount of anti-MHC class I mAb (W6/32) and class II mAb (TC-8B1) for 60 min at 4°C. In c), TcHOT cells were treated with a saturating amount of anti-TCRδ1 (A13), CD3 (HB231), CD4 and CD8 mAbs. The cytotoxicity assays were done for 10 h at an E/T ratio of 25. Bars represent ±SE.

Taken together, these data suggest that BFA perhaps blocks the transport of intracellular peptides from ER to the Golgi apparatus. Consequently, these peptides can not be associated with MHC class I molecules, and cells become resistant to the cytotoxicity of TcHST-2.

BFA effect on the cytotoxicity of TCR γ/δ-type CTL to human autologous tumor Next, we assessed whether BFA could affect the cytotoxicity of TCRδ1$^+$ CTL, TcHOT. As shown in Fig. 4a, this CTL clone was cytotoxic to HOT autologous target, but not to Daudi, K562 and P815 cell lines. This cytotoxicity was not blocked by anti-MHC class I and class II mAbs (Fig. 4b). However, anti-CD3 and TCRδ1$^+$ mAbs partially inhibited the cytotoxicity (Fig. 4c). These data suggest that the cytotoxicity of TcHOT was not MHC-restricted, and that TcHOT may recognize HOT autologous target cells via the CD3-TCRδ1 molecules.

We studied the BFA effect on the cytotoxicity of TcHOT against HOT autologous cells. The cytotoxicity assay demonstrated that BFA clearly inhibited the cytotoxicity between this novel pair of TcHOT CTL and the autologous tumor HOT (data not shown). Furthermore, the effect was cancelled if the reagent was washed out from the culture, consistent with the effect of BFA as seen in TCR α/β-type CTL TcHST-2. Finally, we examined whether the cell surface expression of certain

antigens was influenced by BFA treatment. At least, MHC class I and ICAM-1 expression was not affected. Certain heat shock proteins, such as 70 kDa heat shock cognate protein, were expressed on HOT cells, but this antigen expression was also not influenced by BFA treatment (data not shown).

DISCUSSION

It is well known that $CD8^+$ TCR α/β-type CTL recognizes the antigenic peptides associated with MHC class I molecules.[26-29] This was demonstrated dramatically by using CTL and synthetic peptides specific for the hemagglutinin proteins of influenza A virus[28] and vesicular stomatitis virus.[29] Recently, by using acid elution of the cell extract and reverse-phase HPLC, it was confirmed that naturally processed peptides separated from influenza A virus-infected cells had the same peptide sequence, composed of nine amino acids.[30] Furthermore, there is increasing evidence that MHC class I-restricted CTL can recognize these antigenic peptides, which are derived from the endogenous cellular proteins, and there are several reports suggesting a pathway by which MHC class II-restricted T cells recognize endogenously derived antigens.[13, 31]

During the past five years, the specific destruction of human autologous tumors by CD8 (+) CTL clones has been demonstrated by several laboratories.[1-6] It was shown directly that patients' T cells could be cytotoxic to their own neoplastic cells under certain *in vivo* conditions. In addition, TCR gene rearrangement[32] and PCR analyses using specific primers for TCR Vα gene families[7] showed the usage of certain genes in tumor-infiltrating T cells of melanomas. In our laboratory, preliminary studies by reverse-PCR and nucleotide sequence analysis using primers for TCR Vα[7] and TCR Vβ genes[33] showed that a functional CTL clone, TcHST-2, may use Vα7 or 12, and Vβ20 among the TCR genes (paper in preparation). The data obtained from these experiments led us to investigate the nature of target antigenic peptides recognized by the human autologous tumor-specific CTL.

A fatty acid analogue and a fungal antibacterial reagent, BFA, was first reported by Misumi *et al.*[10] to inhibit the transport of proteins out of the ER. Recently, it has been indicated by Klausner's group that this reagent blocks the transport of peptides from ER to the Golgi apparatus by fusing these two organellas.[15, 16] They also observed strong inhibition of the cytotoxicity of influenza A virus-specific CTL by treating influenza A virus-infected target cells with BFA.[14] This result is consistent with the notion that BFA could prevent presentation of endogenously derived peptides to the MHC class I molecules.

In our present study, in order to determine whether human autologous tumor-specific CTL could recognize the antigen in the BFA-sensitive patheway, we used a $CD8^+$ TCR α/β-type CTL clone, TcHST-2. The data showed that BFA inhibited the cytotoxicity of this clone to HST-2 cells. This effect was cancelled when BFA was removed from the cultures, indicating that the effect of BFA may be reversible. In the reverse ADCC experiment, it appeared that BFA did not markedly affect the cytotoxicity of anti-CD3 mAb-treated TcHST-2 against U937 target cells, although there may have been a very slight inhibitory effect. Accordingly, BFA seems to work at the target cell level, but not at the effector cell level. Furthermore, the inhibition was not seen with chloroquine, which could block exogenous antigen presentation to MHC class II molecules. Moreover, cycloheximide and colchicine, which block the synthesis and elongation of peptides in the ER, and the organization of microtubules, respectively, have no effect on the cytotoxicity of this CTL to the autologous tumor line. The fact that BFA did not influence the cytotoxicity of LAK and NK cells to target tumor cells, including HST-2, also supports our notion that the antigenic peptide of HST-2 cells is recognized by TcHST-2, and that this peptide is processed on a BFA-sensitive intracellular pathway.

Although the cytotoxicity of a CTL clone, TcHST-2, was inhibited by BFA, the same treatment of HST-2 cells with BFA did not cause any change of MHC class I antigen expression on the cell surface. Hsu *et al.* demonstrated a recycling pathway of MHC class I molecules between ER and Golgi apparatus.[34] Although BFA may block this pathway, the fact that BFA did not affect the cell surface expression of MHC class I molecules suggests that MHC class I molecules themselves in the cell surface compartment may play an important role. In fact, it has been demonstrated that MHC class I antigens can recycle on the cell surface.[35] Nuchtern *et al.* also observed that BFA treatment did not influence the cell surface expression of MHC class I molecule of influenza A virus-infected cells.[14] Therefore, our data suggest that 1) the CTL clone's target peptides are possibly endogenously derived from HST-2 autologous tumor cells, and 2) BFA treatment may result in an "emptying out" of the antigenic peptides from MHC class I molecules.

Boon's group[36] and Engelhard's group[37] recently identified the human melanoma antigenic peptides recognized by autologous CTL. Obviously, this kind of research has important implications in the field of human tumor immunology. By using BFA, we are now investigating the nature of the peptide antigens in this autologous system.

BFA also inhibited the cytotoxicity to an autologous human ovarial carcinoma, HOT, of a double negative, TCR γ/δ-type (Vδ1 +) CTL clone, TcHOT. We do

not know the mechanism of cytotoxicity with this novel pair. Our preliminary results suggest that the reverse ADCC of anti-CD3 mAb-bound TcHOT against IgG-Fc receptor (+) U937 target cells was not inhibited by BFA (data not shown). Accordingly, as seen in the TCR α/β-type CTL, TcHST-2, the effect of BFA on the cytotoxicity of TcHOT to HOT tumor cells seems to occur at the target HOT cells, but not at effector cells.

On the other hand, recent reports have shown that heat shock proteins may be expressed on the cell surface,[38] and may participate in the interaction with host T cells, especially with TCR γ/δ-type.[39-42] In these cases, heat shock proteins may be direct ligands for these TCR, or be one of the presenting molecules of antigenic peptides, which might be very different from classical MHC class I and II molecules. This notion is very tempting, since heat shock proteins could bind immature peptides and proteins in an ATP-dependent manner.[43-45] Thus, there is a possibility that BFA is capable of inhibiting the association between these peptides and heat shock proteins. Consequently, in our system, TcHOT may not recognize its target, HOT. We are now examining the validity of this hypothesis by using mAbs to heat shock proteins.

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