An Immunoconjugate of Lys[3]-Bombesin and Monoclonal Antibody 22 Can Specifically Induce FcγRI (CD64)-dependent Monocyte- and Neutrophil-mediated Lysis of Small Cell Carcinoma of the Lung Cells[1]

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ABSTRACT

Small cell carcinoma of the lung (SCCL) accounts for 25% of all lung cancers and has a very poor prognosis. It is known that SCCL cells produce gastrin-releasing peptide, a peptide which has similar biological actions to that of bombesin, an amphibian counterpart of gastrin-releasing peptide, and express high affinity cell surface bombesin/gastrin-releasing peptide receptors. These receptors can serve as targets for specific immunotherapy. Cell surface receptors for the Fc portion of IgG (FcγR) are a family of molecules that can mediate a variety of immune reactions, including tumor cell cytotoxicity. We hypothesized that an immunoconjugate of bombesin and a mAb directed to the high-affinity FcγRI (mAb 22) should be able to trigger specific cytotoxicity against SCCL cells. In this article, we report the construction of this immunoconjugate and demonstrate its capacity to redirect immune effector cells toward SCCL cells and elicit lysis of these target cells. The immunoconjugate stained the majority of cells from four SCCL cell lines and reacted with FcγRI on activated monocytes and neutrophils. After preincubating monocytes and neutrophils with recombinant γ interferon to enhance the expression of FcγRI on the cell surface, we demonstrated that 60–98% of SCCL cells could be lysed in the presence of the immunoconjugate in a chromium release assay. Tumor cell lysis was observed over a wide range of immunoconjugate concentrations, was dependent on the ratio of E:T cells, and could be blocked by the addition of either parental molecule of the immunoconjugate. Bispecific molecules redirecting immune effector cells to target SCCL cells may have clinical application in the therapy of SCCL.

INTRODUCTION

The prognosis for SCCL[3] remains poor despite recent advances in the management of certain other cancers. An estimated 42,500 cases of SCCL were diagnosed in the United States in 1993, accounting for 25% of all lung cancers (1, 2). Although the majority of SCCL patients respond to chemotherapy, the response is of short duration (3, 4). Median survival for patients with limited-stage disease is 14–18 months, and for patients with extensive disease is 9–11 months. Only 15–25% of limited-stage patients survive for more than 2 years (1–4). Many combinations of chemotherapeutic agents have been used clinically without much improvement in the long-term survival rate. Recent efforts have been focused on the development of other therapeutic strategies for the treatment of SCCL, including immunological and hormonal therapy (5, 6) and intensive chemotherapy with autologous bone marrow or peripheral stem cell support (7–9).

BN is a 14-amino acid peptide which was initially isolated from the skin of the frog Bombina bombina (10). The mammalian analogue of BN, GRP, contains a COOH-terminal heptapeptide sequence identical to that of BN (11). The majority of human SCCL cell lines produce GRP and express a single class of high-affinity receptors for BN/GRP (12–16). It has also been demonstrated that GRP functions as an autocrine growth factor for human SCCL cells. Blockage of this autocrine growth stimulatory activity of GRP in SCCL has been the focus of several studies. A mAb against GRP and a number of BN/GRP receptor antagonists have been shown to inhibit the growth of SCCL cells both *in vitro* and *in vivo* (5, 17–19). Since BN/GRP receptors have limited distribution in the body, they can serve as targets for specific immune reactions. A novel approach of immunotherapy targeting the BN/GRP receptors expressed on SCCL cell surface has been developed in our laboratory. We have made an immunoconjugate between a BN-like peptide and a mAb against human high-affinity FcγR (FcγRI, CD64), which is expressed on the surface of human monocytes and activated neutrophils. We hypothesized that this immunoconjugate should

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[3] The abbreviations used are: SCCL, small cell carcinoma of the lung; BN, bombesin; GRP, gastrin-releasing peptide; FcγR, Fc γ receptor; ADCC, antibody-dependent cell-mediated cytotoxicity; SATA, *N*-succinimidyl *S*-acetylthioacetate; Sulfo-SMCC, sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate; R-HPLC, reversed phase HPLC; SH, free sulfhydryl group; rIFN-γ, human recombinant IFN-γ; MFI, mean fluorescence intensity; G-CSF, granulocyte-colony-stimulating factor; PBA, phosphate-buffered saline–bovine serum albumin–

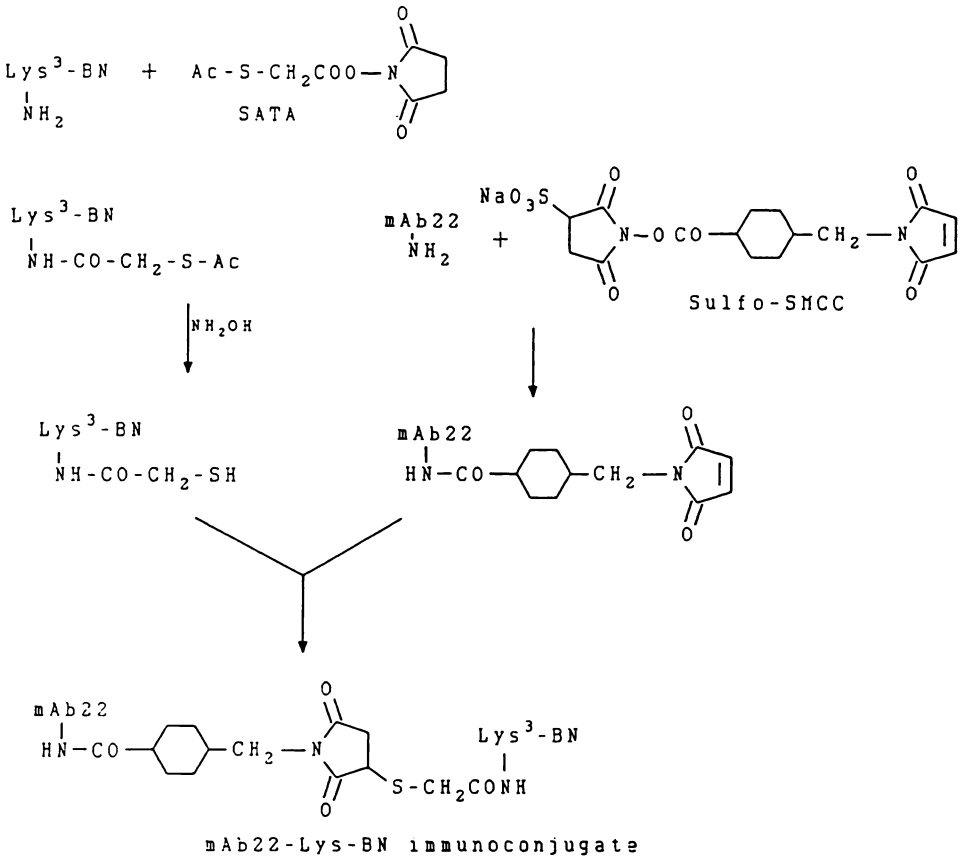

Fig. 1 Schema of the chemical conjugation between Lys^3-BN and mAb 22 or its F(ab′)$_2$ fragments.

be able to redirect these immune effector cells toward SCCL cells and elicit specific ADCC against these cells.

MATERIALS AND METHODS

Cell Lines. SCCL cell lines, NCI-H69, NCI-H345, and SHP-77, were maintained in RPMI 1640 medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO-BRL) at 37°C in a humidified atmosphere with 5% CO_2. Another SCCL cell line, DMS273 (20), was maintained in Waymouth's MB 752/1 medium (GIBCO-BRL) supplemented with 10% FCS.

Antibodies and Reagents. Anti-FcγRI (mAb 22; Ref. 21), F(ab′)$_2$ fragments of mAb 22 (F(ab′)$_2$), and FITC-labeled mAb 22 were obtained from Medarex, Inc. (Annandale, NJ). SCCL-1, an IgG2a mAb reactive with transferrin receptor (22), was produced in this laboratory. Lys^3-BN, a BN analogue with similar binding affinity to the BN/GRP receptor (11, 13), and hydroxylamine were purchased from Sigma Chemical Company (St. Louis, MO). Conjugation chemicals, SATA and Sulfo-SMCC, were obtained from Pierce Chemical Co. (Rockford, IL).

Protein Conjugation. Lys^3-BN was freshly dissolved in 0.1 M sodium phosphate buffer (pH 7.4) containing 2.5 mM EDTA and the SATA was freshly dissolved in 100% dimethylformamide. The SATA was added to Lys^3-BN at a molar ratio of 10:1. After 30 min of reaction at room temperature, the Lys^3-BN-SATA conjugate was separated from nonreacted Lys^3-BN and SATA by R-HPLC on a Vydac C18 analytical column. The R-HPLC eluent containing Lys^3-BN-SATA was adjusted to pH 7.0 by adding 1 M sodium phosphate (pH 8.0). The free sulfhydryl group was generated by deacetylation with hydroxylamine at 4°C for 2 h. A second R-HPLC was performed to separate Lys^3-BN-SH. The fraction containing Lys^3-BN-SH was collected and neutralized to pH 7.0. The presence of free sulfhydryl groups could be determined via reaction with Ellman's reagent. At the same time, mAb 22 or F(ab′)$_2$ fragments of mAb 22 was reacted with Sulfo-SMCC to produce a maleimide-activated antibody. The activated antibody was separated from unreacted Sulfo-SMCC by centrifugation through a Centricon 30 apparatus (Amicon, Beverly, MA). The final conjugation between Lys^3-BN-SH and the activated antibody was carried out by mixing an equal molar amount at room temperature overnight. The unreacted Lys^3-BN and other by-products were removed by centrifugation through a Centricon 30 apparatus. The concentration of the immunoconjugate was quantified using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Richmond, CA) and its purity was checked by SDS-PAGE. The scheme for the peptide conjugation is shown in Fig. 1.

Immunofluorescence. SCCL cells were washed twice with ice-cold PBS containing 0.1% BSA and 0.1% sodium azide

immunoconjugate at 4°C for 1 h in the presence of 100 μg/ml human IgG. The final concentration of immunoconjugate added was 5, 25, and 50 μg/ml per 5×10^6 cells. After washing three times with PBA solution, the cells were resuspended and incubated with FITC-labeled goat $F(ab')_2$ anti-mouse immunoglobulin (Caltag Lab., South San Francisco, CA) for 30 min at 4°C. After washing, the cells were fixed in 1% paraformaldehyde. Monocytes and neutrophils, before and after IFN-γ stimulation, were stained directly with FITC-labeled mAb 22 to evaluate the expression of FcγRI. All of the samples were analyzed by FACScan flow cytometry (Becton Dickinson, San Jose, CA).

The number of antibody sites bound per cell was determined by using a standard bead mixture containing beads with six different concentrations of FITC labeling (23). The standard beads were purchased from Flow Cytometry Standards Corporation (San Juan, Puerto Rico; Quantum 24 and 25). The median fluorescent intensities of the six bead peaks were used to set a standard curve for determination of the amount of FITC/cell. Antibody/cell was calculated by dividing the FITC/cell by the flourescein:protein ratio for the standard bead mixture. Appropriate isotype-matched negative controls were performed to subtract nonspecific binding of antibody.

Stability of Lys^3-BN × 22 Immunoconjugate Binding to SCCL Cells. It has been reported that BN/GRP receptors are rapidly internalized upon binding BN or GRP (24). To determine the stability of immunoconjugate binding, SCCL cells were incubated with 20 μg/ml immunoconjugate for 30 min and then washed to remove unbound immunoconjugate. The immunoconjugate-bound cells were incubated at 37°C in fresh media for various times before the addition of FITC-labeled antimouse antibody at 4°C. The binding of the immunoconjugate to SCCL cells at the initiation of incubation at 37°C was defined as 100%.

Isolation of Effector Cells from Peripheral Blood. Monocytes were separated from Leuko-Packs obtained from the Pittsburgh Central Blood Bank. After Ficoll-Hypaque gradient centrifugation, mononuclear cells were washed twice with HBSS (GIBCO-BRL) containing 1 mM EDTA and then cultured in a flask with RPMI 1640 medium containing 10% FCS for 2 h at 37°C. The nonadherent cells were removed. The adherent cells were detached, collected, stained with anti-CD14, anti-CD45, anti-CD3, anti-CD13, and anti-CD56 (Becton Dickinson), and analyzed by FACScan flow cytometry to determine the purity. The neutrophils were isolated from normal donors. A 20-ml blood sample was centrifuged on Ficoll-Hypaque. The cell pellet was suspended in an equal volume of RPMI 1640 medium. Four hundred μl of 5% dextran were added to each ml of blood sample. After sedimentation at $1 \times g$ for 1 h at 4°C, the neutrophil-rich supernatant was collected. The purity of neutrophils thus isolated was usually higher than 95%.

Activation of Immune Effector Cells. rIFN-γ was kindly provided by Genentech (San Francisco, CA). The concentration of rIFN-γ used in this study (200 units/ml) has been shown to saturate its receptors and to induce a maximum increase in the expression of FcγRI on the surface of monocytes and neutrophils (25). Isolated monocytes and neutrophils were incubated with rIFN-γ in RPMI 1640 medium containing 10% FCS for 18 h at 37°C before the ADCC assay. The expression of FcγRI before and after IFN-γ stimulation was determined by staining with FITC-labeled mAb 22 and analyzed by FACScan flow cytometry.

ADCC Assay. The assay was performed in 96-well round-bottomed microtiter plates (Rainin Instrument Co., Woburn, MA). The target SCCL cells were washed once with RPMI 1640 medium and incubated with 100 μCi sodium [^{51}Cr]chromate (New England Nuclear, Boston, MA) for 1 h at 37°C. After washing several times, cells were resuspended in RPMI 1640 medium containing 10% FCS to a concentration of 1×10^5 cells/ml. Activated monocytes or neutrophils serving as effector cells were suspended in RPMI 1640 medium in a final concentration of 2×10^7 cells/ml. Effector cells (100 μl) were added to the first row of wells and serial dilution was performed with an equal volume of RPMI 1640 medium. Then, 100 μl of target cells were added in the wells to yield a final E:T ratio of 100:1, 50:1, 25:1, and 12:1. In a standard assay, 5 μg immunocongugate were added to yield a final concentration of 25 μg/ml. The mAb SCCL-1 was included in each assay as a positive control to measure the activity of the monocytes and neutrophils. Several other controls with E:T ratio of 100:1 were also incorporated in each assay, including incubation of target cells with immunoconjugate alone, incubation of target and effector cells without any antibody, and incubation of target and effector cells with unconjugated mAb 22. In each assay, a 10-fold excess of Lys^3-BN and unconjugated mAb 22 along with the immunoconjugate were incubated along with the target and effector cells to determine whether the tumor cell lysis could be blocked by either of the parental molecule. To determine whether the presence of human immunoglobulin could interfere with the redirected tumor cell killing, human IgG at a final concentration of 1 mg/ml was added in some assays. After incubation at 37°C for 4 h or 18 h, the microplates were centrifuged and the supernatant was collected for the determination of ^{51}Cr release. Maximum lysis was achieved by the addition of 100 μl 5% NP40 to 100 μl target cells. The percentage of cell lysis was calculated as:

$$(\text{experimental cpm} - \text{spontaneous release mean cpm}) \times 100/(\text{maximum release mean cpm} - \text{spontaneous mean cpm})$$

Spontaneous release of ^{51}Cr from the target cells was less than 10% of maximum release in a 4-h assay and less than 20% in an 18-h assay. For dose-response assays, the immunoconjugate was serially diluted and added. The E:T ratio in those assays was 100:1. Since the amount of immunoconjugate added in a standard assay was 25 μg/ml, we defined the percentage of tumor cell lysis with that amount of immunoconjugate as 100% activity. The tumor cell lysis achieved with diluted immunoconjugate was calculated accordingly.

Statistical Analysis. Each experimental result was obtained from triplicates and reported as the mean ± SD. Significance level was determined by paired Student's t test when applicable.

RESULTS

Binding of the Lys^3-BN × 22 Immunoconjugate to SCCL Cells. Unconjugated mAb 22 and its $F(ab')_2$ fragments

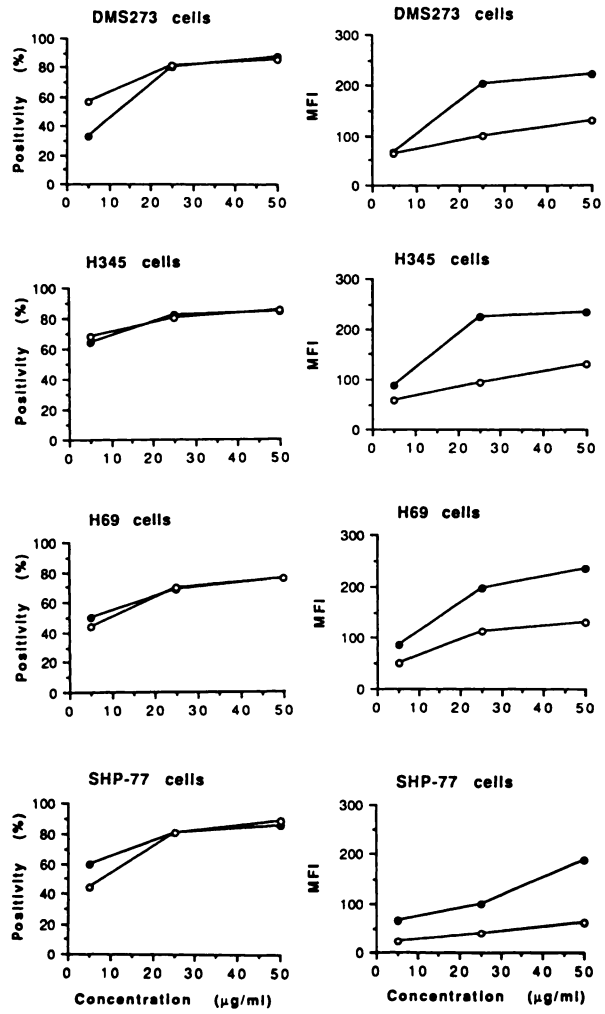

Fig. 2 Flow cytometric analysis of four SCCL lines stained with the immunoconjugate made from Lys^3-BN and mAb 22 (●) or F(ab′)$_2$ (○). Each point represents the mean of three individual analyses and the SD is less than 10% for positivity and 20% for MFI. Isotypic control of mouse IgG was included in all of the analyses. Both the percentage of positive cells and the MFI increase with the amount of immunoconjugate added. Compared with the immunoconjugate made between Lys^3-BN and mAb 22, the immunoconjugate made between Lys^3-BN and F(ab′)$_2$ has a similar percentage of positivity, but consistently lower MFI.

the binding of the immunoconjugate to SCCL cells could be detected by the addition of FITC-labeled antimouse antibody. The results of flow cytometric analysis of the binding of two immunoconjugates to four SCCL cell lines are illustrated in Fig. 2. The binding was directly proportional to the amount of immunoconjugate used to stain the cells. This was manifested both by an increase in the absolute percentage of cells stained positively and by an augmentation of the MFI of the entire cell population. As the amount of immunoconjugate was increased from 5–50 μg/ml, the percentage of positive cells generally increased from 50–85%, and the MFI generally increased from <100 to >200. In general, the immunoconjugate prepared between the F(ab′)$_2$ fragments of mAb 22 and Lys^3-BN had a lower MFI than the one prepared between the F(ab′)$_2$ fragments of mAb 22 and Lys^3-BN. The number of BN/GRP receptors has been estimated to be 1500 and 1000, respectively, for H345 and H69 cell lines (16, 24). We have not quantified the BN/GRP receptor numbers on DMS273 and SHP-77 cells. By flow cytometric analysis, it appears that DMS273 cells express approximately the same number of BN/GRP receptors, while the SHP-77 cells have slightly fewer BN/GRP receptors when compared to H345 cells.

The binding of the Lys^3-BN × 22 immunoconjugate to normal peripheral lymphocytes and to two leukemia cell lines was also tested. The immunoconjugates did not bind to normal peripheral lymphocytes. The mAb 22 and its F(ab′)$_2$ fragments stained 20–50% of cells from the HL-60 and NB4 leukemia cell lines with very dim fluorescence, consistent with the known expression of FcγRI on these cells. There was no change in fluorescence intensity when these cells were stained with the immunoconjugate, although the percentage of positive cells increased slightly.

The number of immunoglobulin molecules per SCCL cell was calculated using a bead calibration method. H345 cells bound an average of 6116 immunoconjugate molecules, H69 cells bound 5036, DMS 273 cells bound 9473, and SHP-77 cells bound 2399. The number of bound immunoconjugates is greater than the published number of BN/GRP receptors per cell (14–16). Since there are three types of BN/GRP receptors present, it is possible that the immunoconjugate may bind to low-affinity BN/GRP receptors or cross-bind to other family members of GRP receptors.

It has been reported that the BN/GRP receptors are quickly internalized upon binding BN/GRP. To determine the stability of Lys^3-BN × 22 immunoconjugate binding, cells from four SCCL lines were incubated with a saturation amount (25 μg/ml) of immunoconjugate for 30 min and then washed to remove unbound immunoconjugate. The cells were incubated at 37°C in fresh media for various times before the addition of FITC-labeled antimouse antibody. The results from three experiments are shown in Fig. 3. The percentage of positive cells did not change significantly during the first 4-h period. After 24-h incubation, the majority of immunoconjugate binding was lost.

Binding of the Lys^3-BN × 22 Immunoconjugate to Monocytes and Neutrophils. We also tested the binding of the Lys^3-BN × 22 immunoconjugate to peripheral monocytes before and after incubation with 200 units/ml rIFN-γ for 18 h. This is shown in Table 1. rIFN-γ dramatically increased the expression of FcγRI on human monocytes as defined by the increase of MFI from <30 to >120. In contrast, there was no change in the expression of FcγRI on human peripheral lymphocytes. The conjugation of Lys^3-BN to mAb 22 did not interfere with its binding to FcγRI. Neutrophils without rIFN-γ treatment did not express cell surface FcγRI or bind to the immunoconjugate. After incubation with rIFN-γ for 18 h, >90% of neutrophils bound to the Lys^3-BN × 22 immunoconjugate with a median MFI of 50.

Lys^3-BN × 22 Immunoconjugate Redirected Tumor Cell Lysis. The ability of the Lys^3-BN × 22 immunoconjugate to elicit monocyte-mediated and neutrophil-mediated tumor cell lysis was tested in a series of ^{51}Cr release assays. The

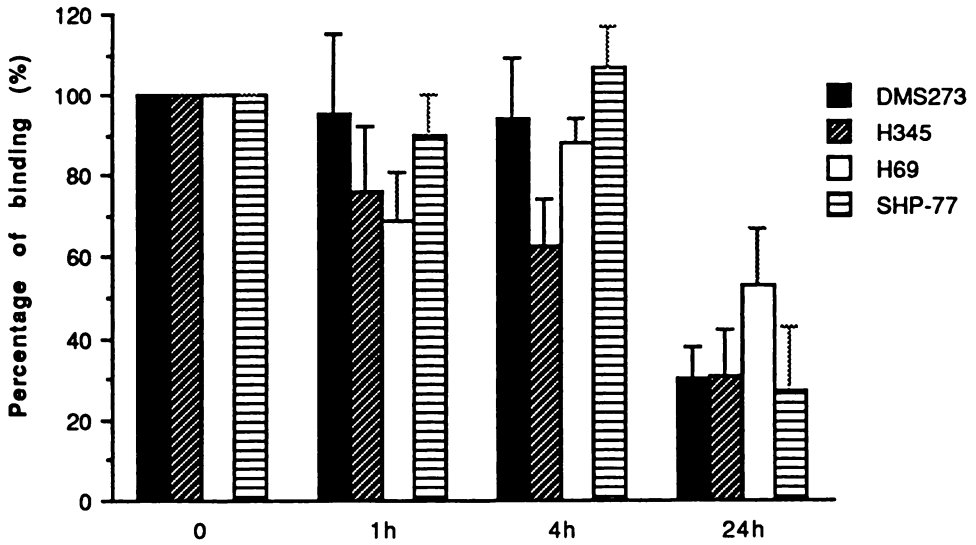

Fig. 3 Stability of the immunoconjugate binding to four SCCL cell lines was analyzed by flow cytometry. The percentage of binding is defined as 100% at the initiation of incubation at 37°C (time 0). Results from three individual experiments are presented as mean ± SD. There is no significant change in immunoconjugate binding to SCCL cells in the first 4 h.

Table 1 Binding of the immunoconjugate to monocytes before and after rIFN-γ incubation[a]

	Before rIFN-γ incubation		After rIFN-γ incubation	
	% Positive ± SD	MFI ± SD	% Positive ± SD	MFI ± SD
mAb 22	83.5 ± 2.2	52.0 ± 26.0 (2)	85.2 ± 16.7	210.1 ± 46.7 (2)
$F(ab')_2$	70.7 ± 15.6	29.9 ± 14.6 (2)	84.7 ± 17.2	124.6 ± 25.5 (2)
mAb 22 × Lys^3-BN	86.7 ± 3.4	25.7 ± 0.10 (2)	92.1 ± 7.60	188.0 ± 85.1 (2)
$F(ab')_2$ × Lys^3-BN	72.3 ± 10.7	26.7 ± 8.57 (2)	85.8 ± 16.3	119.6 ± 20.9 (2)

[a] Flow cytometric analysis of the binding of mAb 22, $F(ab')_2$ of mAb 22, and two immunoconjugates to human monocytes before and after 18-h rIFN-γ incubation. MFI increased significantly after rIFN-γ incubation.

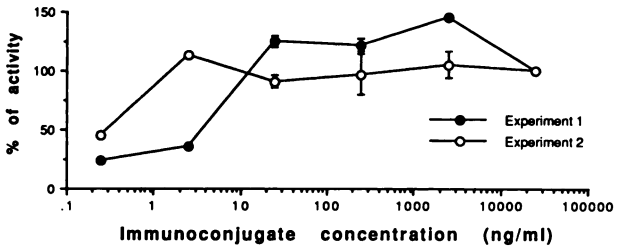

Fig. 4 Effect of different concentrations of immunoconjugate Lys^3-BN × 22 on cytotoxicity of ^{51}Cr-labeled SHP-77 cells by human monocytes at an E:T ratio of 100:1. Results of two experiments are shown. Tumor cell lysis by monocytes achieved with 25 μg/ml immunoconjugate was defined as 100%.

toxicity was determined by a dose-response assay using activated monocytes as effector cells and SHP-77 cells as target cells. Results of two such assays are presented in Fig. 4. Maximum tumor cell lysis was observed over a wide range of concentrations. Since the flow cytometry analysis indicated that the maximum binding of the immunoconjugate to SCCL cells occurred at concentrations above 25 μg/ml, we used 25 μg/ml

The results of monocyte-mediated cytotoxicity of SCCL cells are presented in Fig. 5. Since the effector cells came from different donors in each experiment, the cytotoxic potency varied greatly among donors. For each SCCL cell line, >80% of cells could be lysed by monocytes from some donors. Tumor cell lysis was primarily dependent on E:T ratio in each individual donor. The greatest lysis was consistently achieved at an E:T ratio of 100:1. Pilot experiments using 4-h assays showed moderate cytotoxicity. Increasing the incubation time from 4 h to 18 h resulted in a higher percentage of tumor cell lysis. We also observed that monocytes from the same donor could have different cytotoxic potency against different SCCL cell lines. One SCCL cell line could be better killed by monocytes from one donor, but resistent to monocytes from another donor.

Monocyte-mediated cytotoxicity was studied under different assay conditions in each experiment. The result of a typical experiment is shown in Fig. 6. Incubating target cells with activated monocytes in the absence of immunoconjugate resulted in 15–60% of tumor cell lysis, depending on the donor. The addition of parental antibody, mAb 22, did not further increase the lysis. In the presence of immunoconjugate, tumor cell lysis was increased to 50–95%. This increase is significant

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