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Promotion of natural killer cell growth *in vitro* by bispecific (anti-CD3 × anti-CD16) antibodies

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SUMMARY

Bispecific heteroconjugated $F(ab')_2$ fragments were prepared from pepsin-digested monoclonal OKT3 (anti-CD3) and 3G8 (anti-CD16) antibodies with 5,5′-dithiobis-(2-nitrobenzoic acid). When these bispecific antibodies (BsA) were added to peripheral blood lymphocyte (PBL) cultures with 100 U/ml human recombinant interleukin-2 (rIL-2), preferable growth of natural killer cells occurred. After 3 weeks the frequencies of $CD56^+$ and $CD56^+3^-$ cells in cultures with BsA were $74 \pm 7\%$ and $65 \pm 7\%$, respectively, compared with $48 \pm 6\%$ and $29 \pm 7\%$ in control cultures. The frequencies of $CD3^+$ lymphocytes in the presence of BsA, cells from 1-day cultures were labelled with fluorescein isothiocyanate (FITC)-conjugated anti-CD3, CD4 and CD8 monoclonal antibodies (mAb) and propidium iodide which stains dead cells. Flow cytometry revealed that more than 95% of the dead cells in cultures with BsA were $CD3^+$. Thirty-seven per cent of $CD3^+$, 43% of $CD4^+$ and 17% of $CD8^+$ cells were dead on day 1, and after 3 days the $CD4^+/CD8^+$ ratio among viable lymphocytes was 1·6 in the control and 0·5 in BsA cultures. Taken together, these results show that bispecific (anti-CD3 × anti-CD16) $F(ab')_2$ fragments are strongly immunomodulatory by inducing the killing of T cells by $CD16^+$ cells.

INTRODUCTION

Bispecific antibodies (BsA) are prepared either by chemical, immunological or genetically engineered fusion of two monoclonal antibodies (mAb) with different specificities. BsA have raised interest mainly as tools of targeted immunotherapy.[1] For instance, in the immunotherapy of tumours with killer cells and BsA, one arm of BsA recognizes a triggering structure on lymphocytes (usually CD3) whereas the other arm detects a membrane structure on tumour cells.[2,3] Thereby certain BsA not only guide killer lymphocytes to tumour tissue, but also trigger their cytolytic machinery. The promising clinical effects of BsA detecting CD3 and a glioma antigen (NE150) in the adoptive local treatment of human gliomas have been reported[4] and there are ongoing trials regarding intraperitoneal treatment of ovarian carcinoma patients with killer cells and BsA recognizing CD3 and an ovarian cancer antigen MOv18.[5]

In addition to the targeted immunotherapy, BsA have also been used as experimental immunoregulatory reagents that modify communication between lymphoid cells. For example, enhanced B-cell function through increased helper T-cell effect has been demonstrated by using anti-CD3 × anti-B-cell (anti-$I\text{-}A^b$) BsA.[6]

Human peripheral blood contains different kinds of killer lymphocytes, the major populations being $CD16^+$ $CD3^-$ natural killer (NK) cells and $CD3^+$ $CD8^+$ $CD16^-$ killer T cells. NK cell cytolytic machinery is triggered through CD16 (the low-affinity Fc receptor, FcRIII). CD3 is associated with the T-cell receptor, and is a signal transduction structure. Monoclonal antibodies towards both CD16 and CD3 have been shown to activate NK and T cells, respectively, including their cytolytic functions.[7]

In the present work we have added BsA, which detect the CD16 and CD3 structures to peripheral blood lymphocyte (PBL) cultures. We wanted to analyse the consequences of a close contact between NK cells and T cells in such conditions where the cytolytic machineries of both cell types are activated. The results show that in the presence of anti-CD16 × anti-CD3 BsA, $CD16^+$ cells preferentially lyse $CD3^+$ lymphocytes, especially $CD4^+$ helper cells. These data imply that the killing of autologous T cells by NK cells may, for example, take place in immunodeficiency conditions involving lymphoid cell-specific autoantibodies capable of mediating antibody-dependent cellular cytotoxicity (ADCC) through CD16.

MATERIALS AND METHODS

Peripheral blood lymphocytes

PBL were isolated from buffy coats of venous blood from healthy donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) by Ficoll–Paque (Pharmacia, Uppsala, Sweden) centrifugation and run through nylon wool columns to remove monocytes and B cells.

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Preparation of bispecific antibodies

The mAb OKT3 (anti-CD3) and 3G8 (anti-CD16, FcγRIII) were isolated from hybridoma supernatants using a Prosep A column (Pharmacia). OKT3 hybridoma was purchased from ATCC (Rockville, MD) and 3G8 was kindly provided by Dr J. Ortaldo (NCI, Frederick, MD). The $F(ab')_2$ fragments were isolated from pepsin (Sigma, St Louis, MO) digests[8] by filtration through Diaflo ultrafilter membrane XM50 (Amicon, W.R. Grace & Co., Beverly, MA). BsA were prepared using dithiotreitol (Sigma) as a reducing reagent and thiol-activating reagent 5′,5′-dithiobis-(2-nitrobenzoic acid) (Sigma) according to the published procedure[3], and purified by gel-filtration with Sephadex G-150 (Pharmacia). Purity of the product was tested by SDS–PAGE under non-reducing conditions.

Incubation of cells

The culture medium was RPMI-1640 with 0·29 mg/ml glutamine, 1 mM sodium pyruvate, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Gibco, Paisley, U.K.). The medium was supplemented with 10% heat-inactivated human serum (Finnish Red Cross Blood Transfusion Service) and 100 U/ml of human recombinant interleukin-2 (rIL-2) (EuroCetus B.V., Amsterdam, The Netherlands). One μg/ml of BsA was added to PBL cultures twice, at the beginning and on day 1. Cell concentration was 10^6/ml. Incubation of cells was performed at 37° in humidified air with 5% CO_2.

Phenotyping

For the phenotypic analysis of cultured cells, the following mAb were used: fluorescein isothiocyanate (FITC)-anti-Leu-4 (anti-CD3), FITC-anti-Leu-2a (anti-CD8), FITC-anti-Leu-3a (anti-CD4), phycoerythrin (PE)-anti-Leu-19 (anti-CD56), FITC-anti-TcRαβ (Becton Dickinson, Mountain View, CA), and FITC-anti-TcRγδ (T Cell Sciences, Cambridge, MA). The analysis was performed with FACScan (Becton Dickinson).

Flow cytometry cytotoxicity assay

Cells from PBL cultures were incubated with FITC-conjugated mAb against different T-cell subsets for 30 min on ice. After washing of cells, propidium iodide (PI; Sigma) was added to the cell suspension at a final concentration of 4 μM to determine the percentage of PI-stained, non-viable cells (by measuring the red fluorescence)[9] among T subsets (green fluorescence), using FACScan.

Statistics

Mean values were compared using Student's *t*-test for paired values (StatView 512 program, Brain Power Inc., Calabasas, CA).

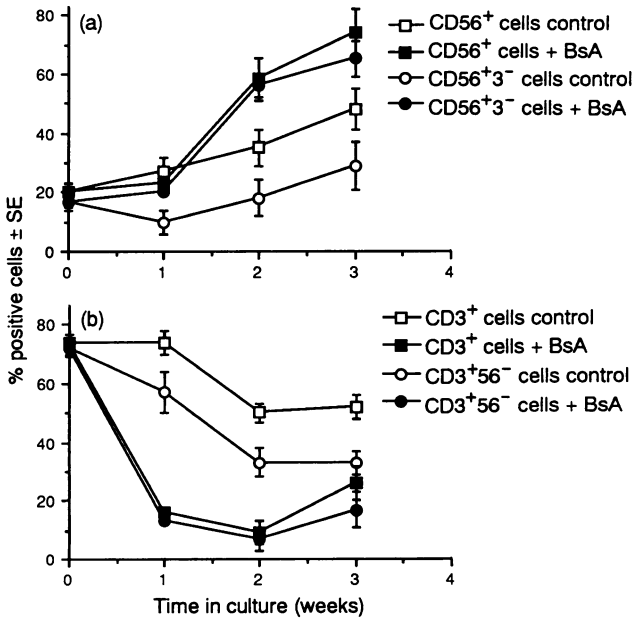

Figure 1. Influence of BsA on $CD56^+$ (a) and $CD3^+$ (b) cell frequencies in PBL cultures. BsA were added to cultures twice, at the beginning and on day 1, at a concentration of 1 μg/ml/10^6 cells. Data from flow cytometry analysis of double-stained (anti-CD56 PE and anti-CD3 FITC mAb) cells are presented as mean ± SE values from five experiments.

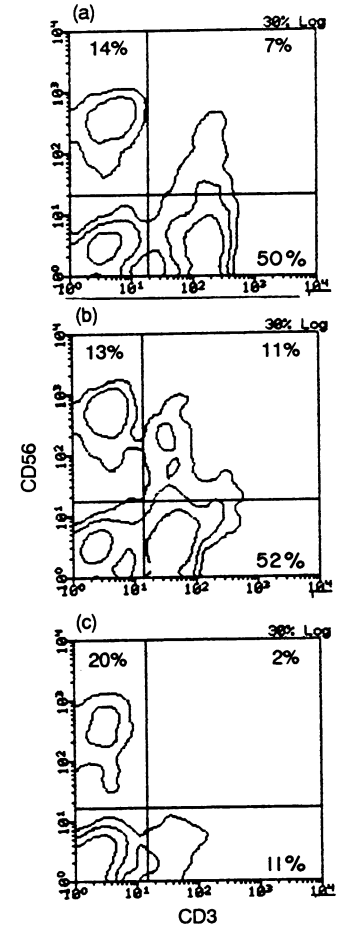

Figure 2. Two-colour immunofluorescence analysis of a 1-week PBL culture. Two-dimensional contour plot of CD3 (green fluorescence, horizontal axis) and CD56 (red fluorescence, vertical axis) expression on lymphocytes incubated with rIL-2 (100 U/ml) without (control, a) and with intact OKT3+3G8 mAb (b) or BsA (c). One representative experiment from three with similar results.

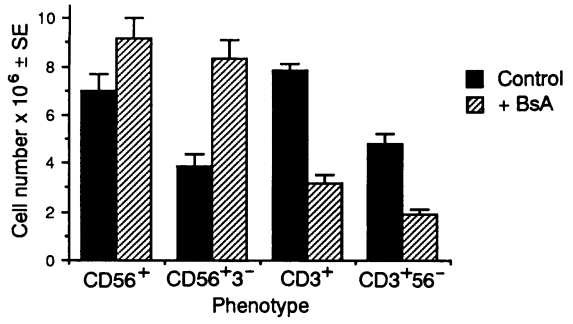

Figure 3. Influence of BsA on $CD56^+$ and $CD3^+$ cell numbers in 3-week PBL cultures. BsA were added to cultures twice, in the beginning and on day 1, at a concentration of 1 mg/ml/10^6 cells. Data from flow cytometry analysis of double-stained (anti-CD56 PE and anti-CD3 FITC mAb) cells are presented as mean ± SE values from five experiments.

RESULTS

Influence of BsA on the phenotype of PBL cultures

The addition of BsA to rIL-2-supported PBL cultures led to a significant increase in the frequencies of $CD56^+$ lymphocytes and $CD56^+3^-$ cells. The frequency of $CD56^+$ cells increased from 23 ± 1 to 74 ± 8% during the second and third weeks of culture (Fig. 1a, Fig. 2). The frequencies of $CD3^+$ and $CD3^+56^-$ (Fig. 1b) and $TcR\alpha\beta^+ + TcR\gamma\delta^+$ cells (not shown) decreased drastically during that time. The total amount of cells was, on average, 20% more in the control cultures on week 3, but the number of $CD56^+$ and especially $CD56^+3^-$ cells had significantly increased in cultures with BsA, together with a corresponding significant decrease of $CD3^+$ cells (Fig. 3).

In subsequent experiments either a mixture of intact OKT3 and 3G8 mAb or BsA was added to cultures. The preferable growth of $CD56^+$ cells was seen only when BsA were present (Fig. 2). $F(ab')_2$ fragments of OKT3 and 3G8 added together did not promote $CD56^+$ cell growth either (not shown).

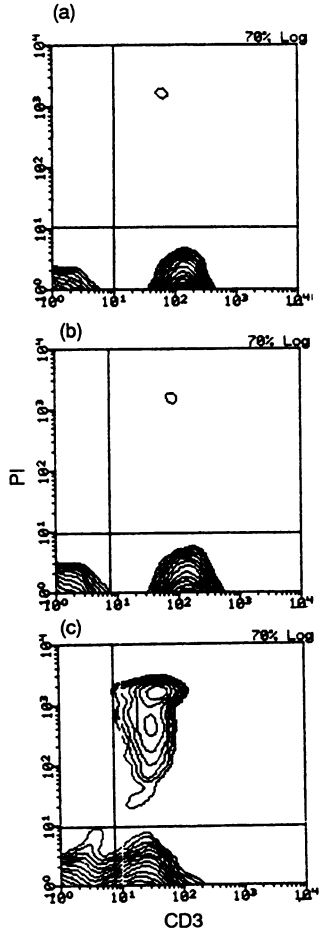

Figure 4. Two-colour immunofluorescence analysis of 1-day PBL culture. Two-dimensional contour plot of CD3 expression on lymphocytes (green fluorescence, horizontal axis) and PI incorporated into non-viable lymphocytes (red fluorescence, vertical axis) incubated without (a) and with OKT3+3G8 mAb (b) or BsA (c). One representative experiment from four with similar results.

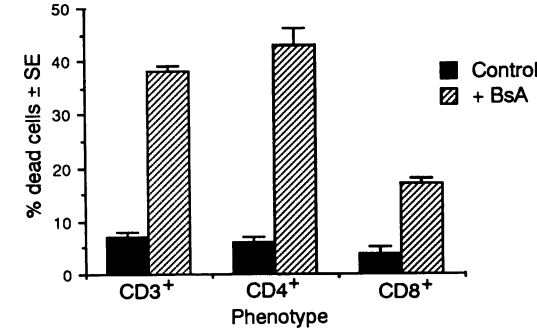

Figure 5. Frequencies of dead cells among different T-cell subsets in 1-day PBL cultures with and without BsA. BsA were added to cultures once at the beginning, at a concentration of 1 μg/ml/10^6 cells. Cells were treated with PI (4 μM/ml) and anti-CD3, anti-CD4 or anti-CD8 FITC-conjugated mAb. Data from two-colour flow cytometry analysis are expressed as mean ± SE values from four experiments.

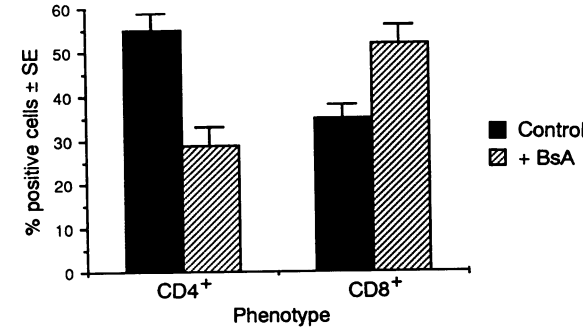

Figure 6. Influence of BsA on the frequencies of $CD4^+$ and $CD8^+$ lymphocytes among viable cells in 3-day PBL cultures. BsA were added to cultures once in the beginning at a concentration of 1 μg/ml/10^6 cells. Cells were treated with PI (4 μM/ml) and anti-CD4 or anti-CD8 FITC-conjugated mAb. Viable cells (not stained with PI) were gated and percentages of $CD4^+$ and $CD8^+$ cells were determined. Data from two-colour flow cytometry analysis are expressed as mean ± SE values from four experiments.

Influence of BsA on cell survival in PBL cultures

Cell viability in 1-day cultures with and without BsA was analysed. In four cultures from different donors, an avarage of 40% of the cells were dead with BsA, as shown by the PI-inclusion test. Ninety-six per cent of all dead cells were $CD3^+$ (Figs 4 and 5). There were not any significant differences between the control cultures (4–5% dead cells) and the cultures to which intact anti-CD3 and anti-CD16 mAb together had been added (5–7% dead cells) (Fig. 4).

In 1-day cultures, the frequency of dead cells among $CD4^+$ cells was twice as much as that among $CD8^+$ cells (Fig. 5). On day 3, the ratio of $CD4^+$ and $CD8^+$ lymphocytes among viable cells was on the average 1·6 in control and 0·5 in BsA cultures (Fig. 6).

DISCUSSION

The results of our study indicate that bispecific mAb which detect CD16 and CD3 induce lysis of CD3-positive cells among peripheral blood lymphocytes cultured in the presence of rIL-2. It is most probable that the killing of T cells by $CD16^+$ NK cells takes place. The $CD4^+$ T lymphocytes were especially sensitive to the killing effect. The lysis of T cells lead to a selective outgrowth of $CD56^+$ $CD3^-$ NK cells.

Since both CD3 and CD16 are triggering structures, one could have expected bidirectional lysis between T cells and NK cells. However, no killing of NK cells by T cells was detectable, although the killing of $CD4^+$ and $CD8^+$ cells by $CD3^+$ T cells in the presence of respective BsA has been described.[10,11] NK cells are the most efficient killer cells in fresh blood. Possibly protective mechanisms against lytic compounds of killer cells are functional in NK cells, due to their basal activation state. An existance of a protective mechanism, operative in killer cells against lytic attack by other cells, is also supported by our finding that $CD8^+$ T cells (including cytotoxic T lymphocytes) were killed less efficiently by $CD16^+$ cells compared with $CD4^+$ helper T cells (Fig. 6).

CD16 is a triggering structure on NK cells and mediates ADCC.[7] On the basis of the present results it may be postulated that, in some immunodeficiency conditions, for instance in AIDS patients, the killing of autologous $CD4^+$ cells by NK cells may take place due to autoantibodies reacting with lymphocyte membrane structures. This may then lead to a converted CD4:CD8 ratio.[12]

In cultures of NK cells, rapid outgrowth of T lymphocytes in the presence of IL-2 is a problem. Bispecific anti-CD3 × anti-CD16 antibodies, through their capacity to induce the lysis of $CD3^+$ cells, may prove helpful in maintaining the purity of NK cell cultures.

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REFERENCES

1. Jung G. & Muller-Eberhard H.J. (1988) A *in-vitro* model for tumor immunotherapy with antibody heteroconjugates. *Immunol. Today*, **9**, 257.
2. Garrido M.A., Perez P., Titus J.A., Valdayo M.J., Winkler D.F., Barbieri S.A., Wunderlich J.R. & Segal D.M. (1990) Targeted cytotoxic cells in human peripheral blood lymphocytes. *J. Immunol.* **144**, 2891.
3. Nitta N., Yagita H., Azuma T., Sato K. & Okumura K. (1989) Bispecific $F(ab')_2$ monomer prepared with anti-CD3 and anti-tumor monoclonal antibodies is most potent in induction of cytolysis of human T cells. *Eur. J. Immunol.* **19**, 437.
4. Nitta T., Sato K., Yagita H., Okumura K. & Ishii S. (1990) Preliminary trial of specific targeting therapy against malignant glioma. *Lancet*, **335**, 368.
5. Mezzanzanica D., Canevary S., Menard S., Pupa S.M., Tagliabue E., Lanzavecchia A. & Colnaghi M.I. (1988) Human ovarian carcinoma lysis by cytotoxic T cells targeted by bispecific monoclonal antibodies: analysis of the antibody components. *Int. J. Cancer*, **41**, 609.
6. Davignon J-L., Vallin-Davignon M., Cohen P.L. & Eisenberg R.A. (1991) Targeting of T-B interaction using heteroconjugate antibody. *Immunology*, **73**, 1.
7. Bolhuis R.L.H. & Braakman E. (1988) Lymphocyte-mediated responses: activation of, and lysis by, cytotoxic lymphocytes. *Curr. Opin. Immunol.* **1**, 236.
8. Parham P. (1983) On the fragmentation of monoclonal IgG1, IgG2a, and IgG2b from BALB/c mice. *J. Immunol.* **131**, 2895.
9. Lebow L.T. & B. Bonavida. (1990) Purification and characterization of cytolytic and noncytolytic human natural killer cell subsets. *Proc. natl. Acad. Sci. U.S.A.* **87**, 6063.
10. Wong J.T., Pinto C.E., Gifford J.D., Kurnick J.T. & Kradin R. (1989) Characterization of the $CD4^+$ and $CD8^+$ tumor infiltrating lymphocytes propagated with bispecific monoclonal antibodies. *J. Immunol.* **143**, 3404.
11. Wong J.T. & Colvin R.B. (1991) Selective reduction and proliferation of the $CD4^+$ and $CD8^+$ T cell subsets with bispecific monoclonal antibodies: evidence for inter-T cell-mediated cytolysis. *Clin. Immunol. Immunopath.* **58**, 236.
12. Daniel V., Schimpf K. & Opez G. (1989) Lymphocyte autoantibodies and alloantibodies in HIV-positive haemophilia patients. *Clin. exp. Immunol.* **75**, 178.