

# An Anti-CD30 Chimeric Receptor That Mediates CD3- $\zeta$ -independent T-Cell Activation against Hodgkin's Lymphoma Cells in the Presence of Soluble CD30<sup>1</sup>

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## Abstract

Hodgkin's lymphoma patients fail to establish an efficient cellular response against CD30<sup>+</sup> Hodgkin/Reed-Sternberg cells. An impaired T-cell receptor/CD3- $\zeta$ -mediated activation of T cells is thought to be involved in this situation. We here present a chimeric anti-CD30 receptor that mediates MHC and T-cell receptor/CD3- $\zeta$ -independent T-cell activation against CD30<sup>+</sup> lymphoma cells even in the presence of soluble CD30. The receptor consists of the binding domain of the monoclonal antibody HRS3 and the signaling unit of the Fc $\epsilon$ RI-receptor  $\gamma$ -chain. After expression in MD45 T cells, receptor cross-linking with immobilized anti-idiotypic monoclonal antibody and CD30<sup>+</sup> cells, respectively, results in increased interleukin 2 secretion and specific cytolysis of CD30<sup>+</sup> Hodgkin's lymphoma cells. Soluble CD30 in concentrations up to 6000 units/ml did not interfere with cellular activation induced by membrane-bound antigen. This demonstrates the feasibility of the chimeric anti-CD30-scFv- $\gamma$  receptor in CD30<sup>+</sup> lymphoma cell targeting, even in the presence of as high concentrations of soluble CD30 as are found in patients during progression of the disease.

## Introduction

Hodgkin's lymphoma is typically composed of a low number of malignant H-RS<sup>3</sup> cells accompanied by a massive infiltration with nonmalignant reactive cells (1). Although HR-S cells express physiological amounts of MHC class I molecules (2), and cytotoxic T cells are present within the tumor tissue (2), the immune system fails to develop an efficient cellular response against H-RS cells. Furthermore, peripheral T cells of patients with Hodgkin's disease were recently reported to have a reduced CD3- $\zeta$  chain expression (3), thus leading to an impaired TCR-mediated cellular activation. This situation stimulates increasing interest in a cell-based immunotherapeutic approach to overcome the MHC restriction and to bypass the deficient TCR/CD3- $\zeta$ -dependent activation. A recently described strategy combines the advantages of T cell- and antibody-based immunotherapy by grafting T cells with chimeric receptors derived from the fusion of the heavy and light chain-variable region of an antibody with an intracellular signaling domain (4–6). The latter mediates activation of cytotoxic T cells directed by the grafted antibody to target cells. The CD30 antigen is a preferred target antigen for the immunotherapy of Hodgkin's disease due to high-density expression on the surface of

H-RS cells of nearly all Hodgkin's lymphoma entities and expression only in moderate densities on the surface of a small subpopulation of normal activated lymphocytes (7). This antigen has, furthermore, been successfully used as a target in antibody-based immunotherapy (8). The application of the anti-CD30 receptor strategy to target Hodgkin's lymphoma cells, however, may be substantially hindered by soluble CD30 frequently found in high concentrations in the serum of Hodgkin's lymphoma patients (9, 10). To resolve this problem, we here present a chimeric anti-CD30 receptor for MHC- and CD3- $\zeta$ -independent T-cell activation and targeting of CD30<sup>+</sup> lymphoma cells that does not interfere with as high concentrations of soluble CD30 as are present in the serum of Hodgkin's lymphoma patients.

## Materials and Methods

**Cell Lines and Antibodies.** MD45 is a mouse CTL hybridoma line (5), L540 is a CD30-positive human Hodgkin's lymphoma-derived cell line (11), and BL60 is a CD30-negative Burkitt's lymphoma line (12). HRS3 is a monoclonal anti-CD30 antibody, and 9G10 is an anti-idiotypic antibody to HRS3 mAb (13, 14). The CD30-Fc fusion protein consists of the extracellular domain of the CD30 antigen, containing the HRS3 epitope, fused to the human IgG1 Fc domain (15).

**Generation of the HRS3-scFv- $\gamma$  Chimeric Receptor.** The variable heavy chain and variable light chain immunoglobulin cDNA sequences of the mAb HRS3 were amplified by PCR by means of immunoglobulin-specific primers (Pharmacia, Freiburg, Germany), and assembled by PCR via a linker coding for a (Gly<sub>4</sub>-Ser)<sub>3</sub> peptide. The assembled product (750 bp) was reamplified by means of oligonucleotides introducing the *Sfi*I and *Nor*I restriction sites, respectively, and ligated into the *Sfi*I and *Nor*I sites of pCANTAB 5E vector DNA (Pharmacia), thereby inserting the scFv DNA in frame with the *M13 gene 3* DNA. Using phage display techniques, recombinant phages were enriched by panning using the anti-HRS3-idiotypic mAb 9G10 and assayed for specific binding to mAb 9G10 by ELISA. The HRS3-scFv DNA sequence was amplified by PCR by means of the following oligonucleotides, introducing an *Xba*I and a *Bam*HI site (underlined), respectively, and a 3' E-Tag sequence: VH5', 5'GCGGCCAGTCTAGAAATGGCCAG3'; E-Tag/VL3', 5'GGTTC-CAGCGATCCGGATACGGC3'. The PCR product was ligated into the *Xba*I and *Bam*HI sites of pRSV- $\gamma$  DNA (5) in frame to the DNA coding for the transmembrane and intracellular part of the human Fc $\epsilon$ RI  $\gamma$ -chain (16, 17).

**DNA Transfection.** DNA (50–100  $\mu$ g) coding for the chimeric receptor was transfected into  $2 \times 10^7$  MD45 T cells by electroporation (one pulse, 250 V, 2400  $\mu$ F) using a gene pulse electroporator (Bio-Rad, Munich, Germany). After 2 days, transfectants were selected in the presence of G418 (2 mg/ml) and subcloned twice by limiting dilution.

**Detection of the Recombinant Receptors.** The recombinant receptor was detected by ELISA as described recently (18). Briefly, nucleus-free cellular lysates of HRS3-scFv- $\gamma$ -transfected cells or B72.3-scFv- $\gamma$ -transfected cells with specificity for TAG72 (18) for control were incubated overnight at 4°C in a microtiter plate (100  $\mu$ l/well) coated with 10  $\mu$ g/ml 9G10 mAb. Bound chimeric receptor molecules were detected with a rabbit antihuman Fc $\epsilon$ RI $\gamma$  antibody (1:5000; Refs. 16 and 17) and an alkaline phosphatase-coupled anti-rabbit immunoglobulin antibody (1:2000; DAKO Diagnostika GmbH,

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<sup>3</sup> The abbreviations used are: H-RS, Hodgkin and Reed-Sternberg; TCR, T-cell receptor; mAb, monoclonal antibody; TAG, tumor-associated glycoprotein; IL-2, interleukin 2.

Hamburg, Germany). The specificity was demonstrated by competition experiments: cell lysates were incubated in the presence of soluble anti-idiotypic mAb 9G10 and mAb HRS3, respectively (both 20  $\mu\text{g/ml}$ ), and the binding inhibition [%] was determined:  $100 \times (1 - \text{binding with competition}/\text{binding without competition})$ . Alternatively, the recombinant receptor was detected by immunoprecipitation and Western blot with the mAb 9G10 and the antihuman Fc $\epsilon$ RI $\gamma$  antibody, as described recently (18).

**Assays for Cellular Activation.** Transfected cells ( $1 \times 10^5$ /well) were cultured for 48 h in microtiter plates with plastic immobilized or soluble 9G10 and an IgG1 control mAb (anti-B72.3), respectively, or were cocultured for 48 h with CD30 $^+$  or CD30 $^-$  lymphoma cells ( $1 \times 10^5$ /well), in the presence or absence of soluble 9G10 and CD30-Fc, respectively. IL-2 in the culture supernatant was determined by ELISA. IL-2 was bound to a solid-phase rat antimouse IL-2 mAb (2  $\mu\text{g/ml}$ ; PharMingen, Hamburg, Germany), detected by a biotinylated rat antimouse IL-2 mAb (0.5  $\mu\text{g/ml}$ ; PharMingen) and a peroxidase-streptavidin-conjugate (1:10,000), and visualized by reaction with 2,2'-azino-di-[3 ethylbenzthiazoline sulfonate (6)] diammonium sulf (both Boehringer Mannheim, Mannheim, Germany). Nontransfected MD45 cells and MD45 cells transfected with the anti-TAG72 chimeric receptor B72.3-scFv- $\gamma$  served as controls (18). Cytolytic activity was measured by time-resolved fluorometry (19). Briefly,  $10^6$  target cells in 1 ml were labeled by electroporation (one pulse, 200 V, 25  $\mu\text{F}$ ) with europium in the presence of 50 mM HEPES, 93 mM NaCl, 5 mM KCl, 2 mM MgCl $_2$ , 2 mM EuCl $_3$ , and 10 mM diethylenetriaminepentaacetic acid (pH 7.4; Merck, Darmstadt, Germany). Effector cells were cocultured for 48 h with glutaraldehyde-fixed L540 cells, washed, harvested, and coincubated with europium-labeled target cells in 96-well round-bottomed microtiter plates ( $1 \times 10^4$ /well) for 6 h at 37°C. Culture supernatants (20  $\mu\text{l}$ /well) were mixed with enhancement solution (200  $\mu\text{l}$ /well; Pharmacia), the fluorescence signals were monitored by time-resolved fluorometry, and the cytotoxicity was calculated as follows:

Cytotoxicity [%] = 100

$$\times \frac{\text{Counts experimental release} - \text{spontaneous release}}{\text{Counts maximal release} - \text{spontaneous release}}$$

Spontaneous release is the europium release in the absence of effector cells. Maximal release is the release obtained by the addition of 1% (v/v) NP40. The

specificity of the cytolytic activity against CD30 $^+$  cells was further demonstrated by cytotoxicity assays in the presence of the anti-idiotypic mAb 9G10 (50  $\mu\text{g/ml}$ ). All cytotoxicity experiments were done in triplicate and analyzed statistically using the Student's *t* test.

## Results and Discussion

The CD30-binding single-chain antibody fragment (scFv) was derived from the HRS3 mAb and isolated by phage display using the anti-HRS3 idiotypic mAb 9G10. The parental mAb HRS3 inhibits binding of the HRS3-scFv to the anti-idiotypic mAb (data not shown), demonstrating that the HRS3-scFv phage antibody retained the same binding specificity with respect to 9G10 mAb as the parental mAb HRS3. No binding was observed to an isotype-matched control antibody with specificity to TAG72 (18). We constructed the chimeric anti-CD30 receptor by fusion of the HRS3-scFv DNA to the cDNA for the transmembrane and intracellular domain of the Fc $\epsilon$ RI receptor  $\gamma$ -chain (16, 17), as described in "Materials and Methods." After transfection into MD45 cells, 14 cell colonies were obtained. The transfected cells react specifically with the anti-HRS3 idiotype mAb 9G10 and the CD30-Fc fusion protein, respectively, as demonstrated by fluorescence-activated cell sorting analysis, whereas the untransfected cells did not (Fig. 1). The chimeric properties of the HRS3-scFv- $\gamma$  receptor molecule were demonstrated by simultaneous binding of the solubilized chimeric receptor molecule to immobilized anti-idiotype mAb 9G10 and detection by an anti-Fc $\epsilon$ RI $\gamma$  antibody (not shown). Binding of the solubilized receptor to immobilized mAb 9G10 was inhibited specifically in the presence of the soluble mAb HRS3 and the soluble anti-idiotype mAb 9G10, respectively, whereas an isotype-matched control mAb did not (not shown). Untransfected MD45 cells do not harbor a protein with these properties. Immunoprecipitation of transfected MD45 clones using mAb 9G10 revealed a protein with a molecular weight of about  $M_r$  43,000 under reducing conditions that reacts specifically with the anti- $\gamma$  antibody (not shown).

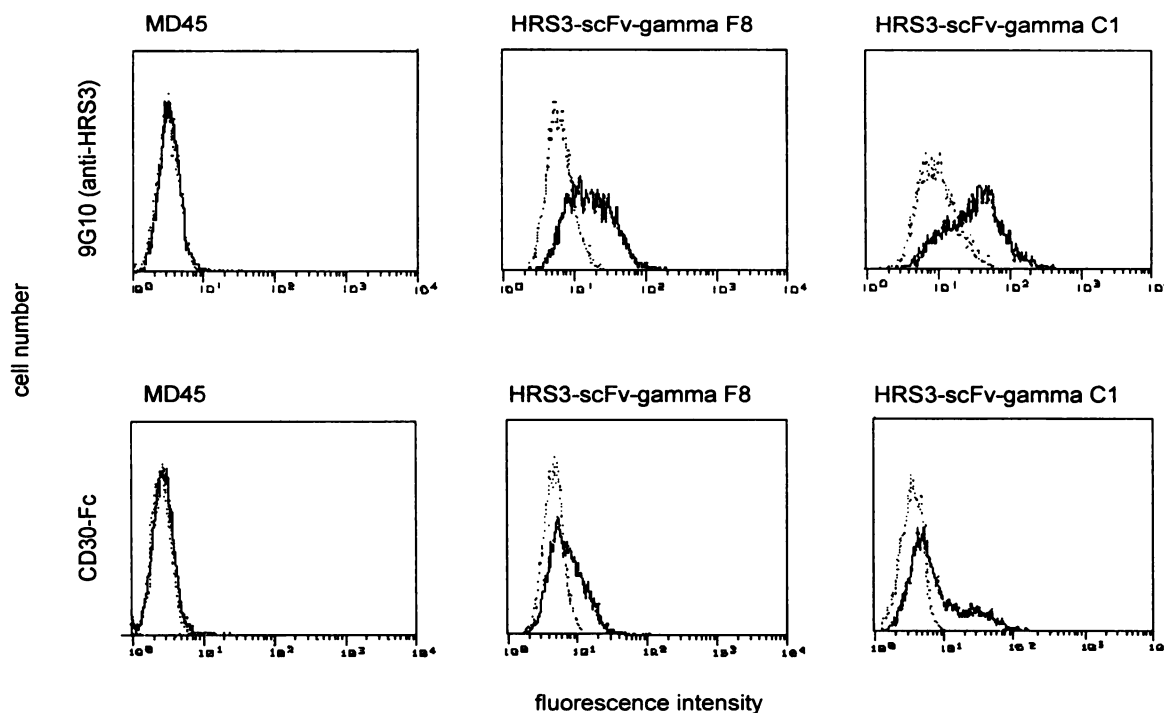
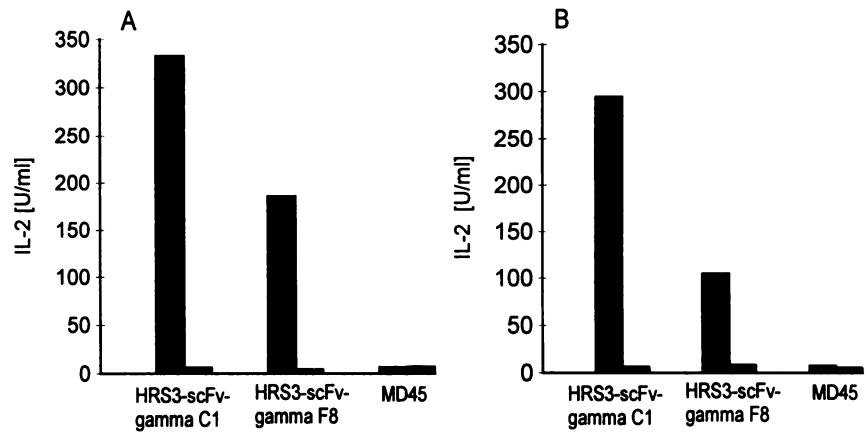


Fig. 1. Expression of the HRS3-scFv- $\gamma$  receptor in transfected MD45 cells. Transfected MD45 cell clones (HRS3-scFv- $\gamma$  C1 and HRS3-scFv- $\gamma$  F8) and untransfected MD45 cells were incubated with the anti-HRS3 mAb 9G10 (5  $\mu\text{g/ml}$ ; top, —) and a murine IgG1 control antibody (5  $\mu\text{g/ml}$ ; top, ····), respectively, and with the CD30-Fc fusion protein (5  $\mu\text{g/ml}$ ; bottom, —) and a human IgG1 control antibody (5  $\mu\text{g/ml}$ ; bottom, ····). After incubation with a FITC-conjugated antimurine and antihuman IgG second antibody, respectively, the cells were analyzed by flow cytometry, and histograms were overlaid.

Fig. 2. IL-2 secretion of HRS3-scFv- $\gamma$ -transfected cells after stimulation with immobilized antigen. A, cells ( $1 \times 10^5$ /well) of two MD45 clones grafted with the chimeric receptor (HRS3-scFv- $\gamma$  C1 and HRS3-scFv- $\gamma$  F8) and of parental MD45 cells were cultured in microtiter plates coated with the anti-HRS3 mAb 9G10 (■) and with an IgG1 control mAb (□), respectively. B, transfected cells ( $1 \times 10^5$ /well) and parental MD45 cells were cocultured with L540 (CD30<sup>+</sup>; ■) and BL60 (CD30<sup>-</sup>; □) tumor cells ( $1 \times 10^5$ /well), respectively. After 48 h, culture supernatants were harvested and assayed for IL-2 by ELISA. The results represent the means of two independent experiments.



Transfected MD45 T cells are activated via the the chimeric receptor after stimulation with CD30 and respond to the alternative FcR- $\gamma$  signaling pathway independently of TCR/CD3- $\zeta$  signaling and in a MHC-unrestricted fashion. To monitor cellular activation after binding of the receptor to antigen, we used induction of IL-2 secretion and specific cytotoxicity to CD30<sup>+</sup> H-RS cells as a marker. Two different cell clones (C1 and F8) grafted with the HRS3-scFv- $\gamma$  receptor were tested and found to secrete increased amounts of IL-2 when cultured in microtiter plates coated with the anti-HRS3 mAb 9G10, but not after incubation in plates coated with an isotype-matched IgG1 mAb (B72.3) with specificity to TAG72 as a control (Fig. 2A). Coculture of these cell clones with L540 (CD30<sup>+</sup>) H-RS cells also resulted in increased IL-2 secretion, whereas coculture of the transfectants with BL60 (CD30<sup>-</sup>) cells did not (Fig. 2B). Untransfected MD45 cells are stimulated neither by the immobilized anti-idiotypic mAb 9G10 nor by CD30<sup>+</sup> or CD30<sup>-</sup> cells, demonstrating that the activation of the transfected cells is mediated by the grafted receptor. The observed differences in IL-2 secretion may reflect intrinsic cell clone variabilities rather than different levels of receptor expression, because the expression levels of the chimeric receptor do not differ substantially between these two cell clones (*cf.* Fig. 1).

The cytolytic activity of MD45 cells grafted with the HRS3-scFv- $\gamma$  receptor was assayed by cocubation with L540 (CD30<sup>+</sup>) Hodgkin's lymphoma and with BL60 (CD30<sup>-</sup>) Burkitt's lymphoma cells as a control. Receptor-grafted MD45 cells exhibited cytotoxicity to CD30<sup>+</sup> L540 cells but not to CD30<sup>-</sup> BL60 cells, whereas the untransfected MD45 cells did not (Fig. 3). The degree of specific cytolysis depends on the E:T ratio. Preincubation of the transfected MD45 cells with the anti-HRS3 mAb 9G10 (50  $\mu$ g/ml) for 30 min completely blocked the cytotoxicity to CD30<sup>+</sup> cells (data not shown), demonstrating that the cytolytic activity of HRS3-scFv- $\gamma$  receptor-

grafted cells to CD30<sup>+</sup> tumor cells is due to specific interaction with the anti-CD30 receptor.

High serum levels of soluble CD30 (65–2000 units/ml) are frequently found in patients with Hodgkin's disease (9, 10). To determine whether soluble CD30 in those concentrations blocks the chimeric receptor, we conducted stimulation experiments in the presence of soluble CD30-Fc fusion protein and the anti-idiotypic mAb 9G10, respectively, and compared their blocking activity. The anti-idiotypic mAb 9G10 was assayed because of high-affinity binding to the HRS3-scFv domain of the receptor. The CD30-Fc fusion protein was standardized by ELISA for the determination of serum CD30 (20). Transfected MD45 cells were cocubated for 48 h with L540 (CD30<sup>+</sup>) cells in the presence of increasing amounts of anti-idiotypic mAb 9G10 and of soluble CD30, respectively. Whereas mAb 9G10 efficiently inhibits IL-2 secretion of transfected MD45 cells, soluble CD30 did not, even in concentrations of 11  $\mu$ g/ml (equivalent to 6600 units/ml; Fig. 4A). The differences between soluble mAb 9G10 and CD30-Fc in blocking the chimeric receptor may reflect different binding affinities for the HRS3-scFv receptor domain. We investigated whether or not soluble CD30 activates HRS3-scFv- $\gamma$ -transfected cells, which would result in nonspecific activation at nontumor sites. Transfected cells were stimulated with soluble CD30-Fc fusion protein and mAb 9G10, respectively. As shown in Fig. 4B, the anti-idiotypic mAb 9G10 activates transfected cells to some extent, whereas soluble CD30 and an isotype-matched IgG1 mAb (B72.3) did not. Cross-linking of the receptor by cocubation with CD30<sup>+</sup> tumor cells, however, induced a nearly 10 times higher IL-2 secretion than after incubation with the soluble anti-idiotypic mAb. This indicates that the immobilized antigen, in contrast to soluble CD30 or soluble anti-idiotypic mAb, preferentially initiates cellular activation by the HRS3-scFv- $\gamma$  receptor. Cross-linking of the chimeric anti-CD30 re-

Fig. 3. Specific cytotoxicity of HRS3-scFv- $\gamma$  receptor-transfected MD45 cells to L540 (CD30<sup>+</sup>) Hodgkin's lymphoma cells. Two HRS3-scFv- $\gamma$  receptor-transfected MD45 cell clones (HRS3-scFv- $\gamma$  C1, ●; HRS3-scFv- $\gamma$  F8, ■) and untransfected MD45 cells (▲) were preincubated with glutaraldehyde-fixed L540 (CD30<sup>+</sup>) tumor cells for 48 h. The cells were harvested and washed, and the cytolytic activity against europium-labeled L540 (A) and BL60 (B) cells ( $1 \times 10^4$  cells/well), respectively, was determined after 6 h by time-resolved fluorometry. The results represent the means of triplicates; bars, SE.

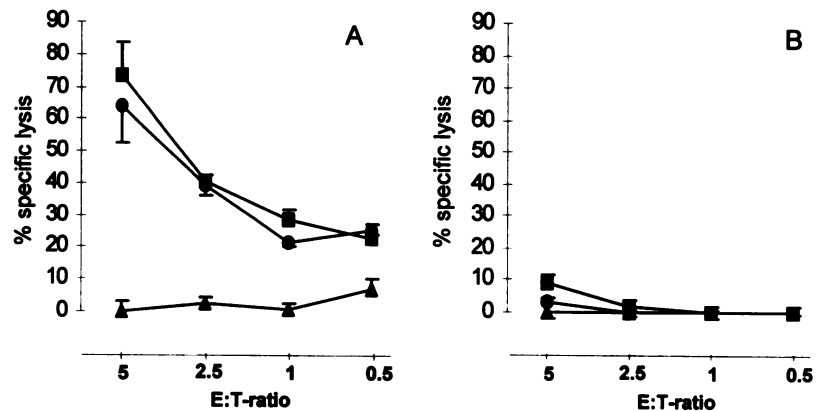
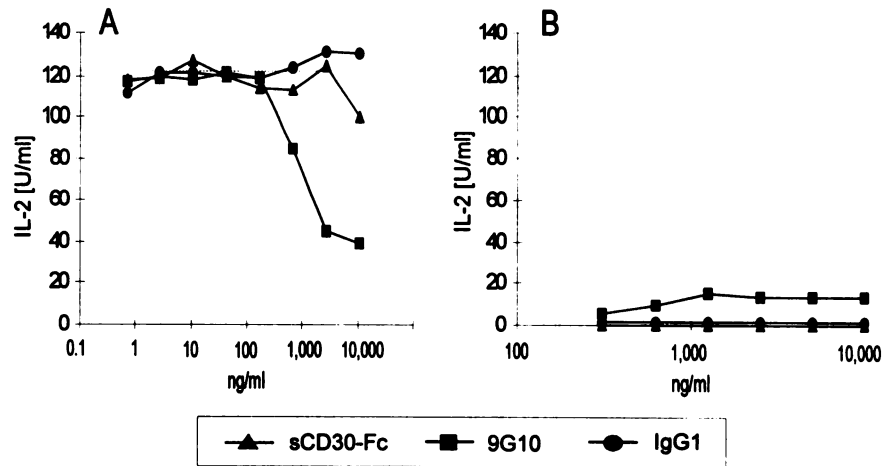


Fig. 4. IL-2 secretion of anti-CD30 receptor grafted MD45 cells upon or without coincubation with L540 (CD30<sup>+</sup>) lymphoma cells in the presence of soluble CD30 antigen and of the anti-idiotypic mAb 9G10. A, HRS3-scFv- $\gamma$  receptor-transfected MD45 cells (clone F8;  $1 \times 10^5$  cells/well) were cocultured with L540 (CD30<sup>+</sup>) cells ( $1 \times 10^5$  cells/well) in the presence of increasing amounts of the indicated mAbs and of soluble CD30-Fc protein, respectively. B, HRS3-scFv- $\gamma$  receptor-transfected MD45 cells (clone F8;  $1 \times 10^5$  cells/well) were incubated in the presence of different amounts of the indicated mAbs and of soluble CD30-Fc protein, respectively. After 48 h, supernatants were harvested and assayed for IL-2 by ELISA. A and B,  $\cdots$ , amount of IL-2 secreted by HRS3-scFv- $\gamma$  receptor grafted cells (clone F8) in the presence of CD30<sup>+</sup> tumor cells without coincubation of mAb 9G10 and soluble CD30-Fc protein, respectively.



ceptor by highly complexed antigen is obviously required for efficient cellular activation. Accordingly, soluble anti-idiotypic mAb 9G10 only slightly activates, and soluble CD30 nearly completely fails to activate, HRS3-scFv- $\gamma$  receptor-grafted cells.

We think these observations are of substantial relevance with respect to clinical applications, because high levels of soluble CD30 antigen in sera of Hodgkin's lymphoma patients would block an efficient tumor cell targeting by a chimeric anti-CD30 receptor. The mean serum values of soluble CD30 antigen are in the range of 65–458 units/ml during progression of the disease, with individual serum levels up to 2000 units/ml (9). The concentration of soluble CD30 that partially interferes with activation of anti-CD30-scFv- $\gamma$  receptor-expressing cells in the presence of CD30<sup>+</sup> tumor cells, however, is about 15-fold higher (*i.e.*, 6600 units/ml, corresponding to 11  $\mu$ g/ml) than the mean serum values (*cf.* Fig. 4A). Even this concentration of soluble CD30 antigen does not mediate cellular activation in the absence of CD30<sup>+</sup> tumor cells. We therefore expect that soluble CD30 will neither nonspecifically activate nor block activation of receptor-grafted cells by immobilized or cell-bound CD30, making the HRS3-scFv- $\gamma$  receptor suitable for MHC-unrestricted and TCR/CD3- $\zeta$ -independent targeting of T cells to CD30<sup>+</sup> Hodgkin's lymphoma cells, even in the presence of high levels of soluble CD30.

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