

Retrobulbar T Cells from Patients with Graves' Ophthalmopathy Are CD8⁺ and Specifically Recognize Autologous Fibroblasts

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Abstract

Graves' ophthalmopathy is an autoimmune condition characterized by T cell infiltration of the retrobulbar tissue. Phenotypic and functional analysis of these infiltrating cells may provide insight into the pathogenesis of the disease. IL-2-responsive cells were therefore grown out of the retrobulbar tissue from two patients with severe Graves' ophthalmopathy undergoing orbital decompression surgery, and six T cell lines were established and characterized. They consisted predominantly of CD8⁺CD45RO⁺ cells and secreted IL-4, IFN- γ , and IL-10 upon activation. When screened for their antigen reactivity, all lines proliferated in response to stimulation with autologous retrobulbar fibroblasts in an HLA class I-restricted manner, but did not recognize autologous peripheral blood mononuclear cells, crude eye muscle extract, allogeneic cells, or purified protein derivate of *Mycobacterium tuberculosis*. In contrast, PBMC from the same patients responded readily to purified protein derivate of *Mycobacterium tuberculosis* and allogeneic PBMC, but did not recognize autologous fibroblasts. Interestingly, only one of the six retrobulbar T cell lines displayed cytotoxicity towards its specific target cell population. These results suggest that the retrobulbar fibroblasts are a major T cell target in Graves' ophthalmopathy. Pronounced cytokine production in the absence of target cell cytotoxicity may explain fibroblast proliferation, glycosaminoglycan secretion, and secondary eye muscle enlargement in this condition. (*J. Clin. Invest.* 1994. 93:2738–2743.) Key words: Graves' disease • ophthalmopathy • T lymphocytes • fibroblasts • cytokines

Introduction

Graves' disease hyperthyroidism is frequently accompanied by ophthalmopathy (1). The clinical picture of this condition ranges from discomfort and lid retraction to disfiguring proptosis, diplopia, and sight loss. No completely satisfactory treatment is at present available, and the etiology and pathogenesis of the disorder are still poorly understood (2, 3). Histological

examinations of retrobulbar tissue samples demonstrate a diffuse and focal mononuclear cell infiltration of the connective tissue primarily by activated T cells with a few B cells, macrophages, and mast cells present (4, 5). Among T cells there is a predominance of CD8⁺ cells (4). This infiltration is accompanied by activation of the surrounding fibroblasts, which in contrast to the muscle cells express MHC class II molecules (5). Fibroblasts are numerous, and there is excessive deposition of extracellular matrix proteins, notably collagen and glycosaminoglycans (6–9). Apart from subsarcolemmal deposits of lipid and glycogen, the extraocular muscle fibers appear strikingly normal, with no histological evidence of destruction (7, 9).

Early results, which demonstrated the presence of autoantibodies against eye muscle antigen in sera from patients with Graves' ophthalmopathy (GO)¹ (10, 11), had led to the original concept that the disorder was due to an immune response against the extraocular eye muscle fibers. However, this hypothesis was weakened by reports demonstrating that the autoantibodies in question were neither tissue nor disease specific (12). Such data, as well as the lack of histological abnormalities of extraocular eye muscle fibers combined with the marked fibroblast activity observed in pathological specimens from GO patients, formed the basis for a new concept, which suggested that orbital fibroblasts were instrumental in the pathogenesis of the disease (13). Autoantibodies against 23- and 64-kD fibroblast antigens were found in the majority of patients with GO (14, 15), and GO IgGs were capable of stimulating collagen production by fibroblasts, a property shared with some monoclonal antibodies to the thyroid-stimulating hormone receptor (16). Graves' disease retroorbital fibroblasts were also proposed to possess unique immunogenic properties, such as the constitutive expression of MHC class II molecules (17) and of the normally stress inducible heat shock protein hsp 72 (18). Both qualities might support fibroblast recognition by T cells (19, 20). Although obviously critical for the initiation of an autoimmune response, little is still known about the role of T cells in GO. Recent work demonstrated that retrobulbar T cells had a Th1-like cytokine profile and were able to display non-specific cytotoxicity (21). However, antigen reactivity studies until now have been confined to circulating T cells (22, 23), which only imprecisely, if at all, reflect the responses of cells at the site of an autoimmune event. The present report is, to our knowledge, the first one on autoreactive T cells grown out of retrobulbar tissue from patients with GO. We demonstrate that

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1. Abbreviations used in this paper: GO, Graves' ophthalmopathy; OKT3, anti-CD3 antibody; PPD, purified protein derivate of *Mycobacterium tuberculosis*.

retrobulbar T cells specifically recognize, but do not necessarily kill, autologous retroorbital fibroblasts.

Methods

Patients. Retrobulbar tissue specimens were obtained from two patients (one male, one female; age: 40 and 24 yr, respectively) with severe GO undergoing orbital decompression surgery. Both patients (E.P. and C.H.) had a long standing history of Graves' hyperthyroidism. One had undergone subtotal thyroidectomy in the past, but had suffered several relapses of thyrotoxicosis afterwards. The other one had had recurrent hyperthyroidism for 6 yr. Both patients had failed to respond to glucocorticoid treatment. At the time of decompression surgery they were both euthyroid, one without any medication, the other one treated with low doses of methimazole.

Tissue samples and cell purification. Connective and eye muscle tissue samples were obtained under sterile conditions at surgery and transported on ice to the laboratory. From each sample, small blocks were shock frozen and stored in liquid nitrogen. These samples were later used as a source of crude antigen. From the remaining tissue, isolated cells were obtained according to the following protocol. Retroorbital tissue samples were digested with collagenase type IV (Sigma Chemie GmbH, Deisenhofen, Germany) as described previously for autoimmune thyroid tissue (19). The obtained cell suspensions were left in tissue culture flasks overnight in RPMI supplemented with 10% decompartmented FCS (Gibco Laboratories, Grand Island, NY). The nonadherent infiltrating cells were then removed by washing, enriched by Ficoll-Paque (Pharmacia AB, Uppsala, Sweden) gradient centrifugation, and cultured as described below. The adherent cell population, which consisted of a mixture of fibroblasts and eye muscle cells, was kept in RPMI, 10% FCS to grow into continuous lines. Muscle cells did not grow under the culture conditions used and were no longer present after several passages. The remaining cell population was identified as a 100% pure fibroblast population by staining with an antibody against collagen type III (kindly provided by R. Timpl, Munich, Germany). Fibroblasts were removed from their plastic support by a 5–10-min exposure to trypsin EDTA (trypsin, 1:250; 2 g/liter EDTA in modified Puck's saline; Gibco Laboratories), frozen down, and kept in liquid nitrogen until later usage in T cell proliferation/cytotoxicity assays. From one of the patients, who had given consent, control fibroblasts could be obtained from a skin biopsy.

Preparation of T cell lines. T cell lines were prepared from the nonadherent cell population as described previously for intrathyroidal cells (19). In brief, *in vivo* activated T cells were selected by culture for 10 d in IL-2 (20 ng/ml) (Sandoz, Vienna, Austria) only, followed by further stimulation with anti-CD3 antibody (OKT3) (American Type Culture Collection, Rockville, MD), which has been shown to stimulate growth while retaining antigen specificity (24). OKT3 was added to the cell lines together with irradiated (40 Gy) autologous PBMC as feeders every 7 d, and IL-2 (20 ng/ml) was added every 3 d. T cells were separated from contaminating fibroblasts every week over the first 2 wk of culture. Three parallel cultures were established from each patient.

Preparation of PBMC. PBMC were isolated from heparinized blood by centrifugation over a Ficoll-Paque gradient and washed twice.

Immunofluorescence staining. For the phenotyping of lines, cells were transferred into round bottom tubes (Becton Dickinson Immunocytometry Systems, Mountain View, CA) (10^5 cells/tube) and washed in PBS containing 0.1% BSA at 4°C (Janssen, Olen, Belgium). FITC-conjugated monoclonal antibodies against CD4, CD8, CD45-RA, and CD45RO (Becton Dickinson Immunocytometry Systems and Immunotech, Marseille, France) were then added, and the cells were incubated for 40 min at 4°C. Thereafter, cells were washed twice in PBS at 4°C and analyzed on a fluorescence-activated cell sorter (Becton Dickinson Immunocytometry Systems). 5,000 scatter-gated cells were analyzed in each sample. The frequency and fluorescence profile of the cells were determined with logarithmic signal amplifiers.

Preparation of supernatants for cytokine secretion analysis. Cells were seeded at a density of 10^6 in 48-well plates (Costar Corp., Cam-

bridge, MA), which had been coated previously with OKT3 in RPMI, 10% FCS supplemented with IL-2 (20 ng/ml). Duplicate cultures were performed. Incubation was carried out in a total supernatant volume of 1 ml. After 48 h, the cultures were harvested and centrifuged at 250 g, and the supernatants were stored at -80°C until assayed for the presence of cytokines.

Cytokine determinations. The levels of IL-4, IL-10, and IFN- γ were determined in sandwich ELISAs as described recently (25).

Proliferation assays on T cell lines. T cells were cultured at 5×10^4 cells/well in 96-well flat-bottomed plates in RPMI containing 10% normal human decompartmented serum (PAA Labor und Forschungs Ges.M.B.H., Linz, Austria). Triplicate cultures were performed in each experiment. T cell proliferation in response to the following stimuli was investigated: irradiated autologous and allogeneic retrobulbar fibroblasts (10^4 cells/well; 40 Gy), irradiated allogeneic PBMC (10^5 cells/well; 40 Gy) alone and in combination with purified protein derivative of *Mycobacterium tuberculosis* (PPD) (Behring Institute, Vienna, Austria) (100 $\mu\text{g}/\text{ml}$), homogenized autologous fibroblasts, or homogenized eye muscle (1:10 and 1:100 dilutions). For blocking experiments, autologous fibroblasts were used after the addition of a monoclonal antibody against the monomorphic determinant of HLA class I (W6/32; kindly provided by D. Schendel, Munich, Germany) (1 $\mu\text{g}/\text{ml}$). After a 48-h incubation period, cultures were pulsed with [^3H]thymidine (1 $\mu\text{Ci}/\text{well}$) (Amersham International, Buckinghamshire, UK) for 16 h. Cells were then harvested as described previously (19). Results were expressed as counts per minute.

Proliferation assays on PBMC. PBMC were cultured at a density of 10^5 cells/well. Culture conditions were the same as described for T cell lines. PBMC were incubated for 6 d. Proliferation was then assessed as described for T cells.

Cytotoxicity assay. Fibroblasts were incubated for 1 h at 37°C with 100 μCi sodium chromate (Amersham International) in RPMI, 5% human serum. The cells were then washed three times, and their concentration was adjusted to give 10^4 cells/test. T cells were added at target/effector cell ratios of 1:10 and 1:100. Duplicate cultures were performed. Cocultures were left to incubate for 4 h at 37°C . Afterwards, the tubes were centrifuged at 400 g for 5 min, and 100 μl aliquots of each supernatant were counted for 1 min on a gamma counter. The percent cytotoxicity was calculated by: $([\text{experimental cpm} - \text{spontaneous cpm}]/[\text{maximal cpm} - \text{spontaneous cpm}]) \times 100\%$. Spontaneous release was defined as the radioactivity released by fibroblasts cultured in the absence of T cells. Maximal release was defined as the radioactivity released by labeled fibroblasts after repeated freezing and defrosting.

Results

Phenotyping and cytokine production of expanded retrobulbar T lymphocytes. Parallel cultures of T cells were established from the retrobulbar tissue of two patients with GO. Histological examination of tissue sections had demonstrated the hallmarks of retrobulbar pathology in GO, i.e., severe edema, fibroblast proliferation, and the presence of muscle fibers. In both instances, massive interstitial mononuclear cell infiltration, located predominantly between fibroblasts and less prominently between fat cells and within muscle tissue, was observed. Immunohistological investigations showed the majority of these cells to be CD3 $^+$ (not shown). T cells responded and increased well in number upon stimulation with IL-2 and anti-CD3. After 4 wk, sufficient cells were available for analysis. All six lines consisted predominantly of T cells of the CD8 phenotype (Table I). The percentage of CD4 $^+$ helper/inducer cells ranged between 3 and 20% and was $< 10\%$ in five of the lines. Low numbers of CD4 $^-$ CD8 $^-$ and of CD4 $^+$ CD8 $^+$ cells were detected

Table I. FACS® Analysis of Retrobulbar T Cell Lines from Two Patients with GO

| T cell line | EP 3 | EP 4 | EP 7 | CH 1 | CH 2 | CH 3 |
|---------------------------------------|------|------|------|------|------|------|
| CD4 ⁺ (%) | 3 | 20 | 4 | 9 | 4 | 9 |
| CD8 ⁺ (%) | 90 | 77 | 93 | 89 | 89 | 96 |
| CD4 ⁻ CD8 ⁻ (%) | 6 | 1 | 2 | 0 | 6 | 2 |
| CD4 ⁺ CD8 ⁺ (%) | 1 | 2 | 1 | 2 | 1 | 4 |
| CD45RA ⁺ (%) | 0 | 0 | 0 | 0 | 0 | 0 |
| CD45RO ⁺ (%) | 100 | 100 | 100 | 100 | 100 | 100 |

Cells were stained with FITC-conjugated antibodies against CD4, CD8, CD45RA, and CD45RO. Results represent the percentage of positive cells in the different lines.

in all samples. All T cell lines were CD45RO⁺/RA⁻. Upon activation, cytokines were secreted into the culture medium. The cytokine secretion pattern was similar in the three T cell lines investigated (Table II). They all secreted relatively high amounts of IFN- γ and IL-10. Two of the lines also produced considerable quantities of IL-4, whereas one line had lower but still clearly detectable IL-4 production. Retrobulbar fibroblasts secreted none of the cytokines analyzed.

Retrobulbar T lymphocytes proliferate in response to stimulation with autologous retrobulbar fibroblasts. When screened for their capacity to proliferate in response to stimulation with potential autoantigens, all six lines gave a marked proliferative response upon stimulation with autologous retrobulbar fibroblasts (Fig. 1). The magnitude of the response ranged between 427 and 4,352%, when background proliferation was considered as 100%. This response was HLA class I restricted, as it could be blocked by addition of an antibody against the monomorphic region of HLA class I. Two of the lines were also screened for their proliferation upon stimulation with homogenized fibroblasts added in combination with irradiated autologous PBMC. A positive response was noted in both lines. No stimulation was observed when retrobulbar T cells were cocultured with allogeneic mismatched fibroblasts or with other autologous cells such as irradiated PBMC. The effect of a crude eye muscle preparation added in combination with autologous irradiated PBMC as antigen-presenting cells was analyzed in three of the lines, but was unable to induce T cell proliferation in any of the lines tested. Retrobulbar T cell lines also recognized autologous skin fibroblasts. However, this response was less pronounced than the one against retrobulbar fibroblasts in all three lines tested (Table III).

Table II. Cytokine Secretion by Retrobulbar Cell Lines from Patients with GO

| | Line EP 3 | Line CH 1 | Line CH 2 | Fibroblasts EP |
|---|-----------|-----------|-----------|----------------|
| IL-4 (pg/10 ⁶ cells per 24 h) | 673 | 535 | 25 | ND |
| IFN- γ (pg/10 ⁶ cells per 24 h) | 5380 | 2893 | 3025 | ND |
| IL-10 (pg/10 ⁶ cells per 24 h) | 1040 | 220 | 600 | ND |

T cell lines were activated by OKT3 coated to plastic and left to incubate in RPMI/10% FCS supplemented with IL-2 (20 ng/ml). Fibroblasts were kept in RPMI/10% FCS. The duration of the culture was 48 h. ND, not detectable.

Retrobulbar T cell lines do not derive from contaminating peripheral cells. To exclude the possibility that retrobulbar T cell lines were derived from peripheral passenger T cells, proliferation of retrobulbar T cell lines and proliferation of PBMC from the same patients were compared. Whereas PBMC from both patients responded readily to PPD and to allogeneic irradiated PBMC, neither of the two antigens was stimulatory on retrobulbar cells (Table IV). In contrast, no proliferation was observed when PBMC were cocultured with autologous retrobulbar fibroblasts, demonstrating that fibroblast-specific T cells were restricted to the retrobulbar tissue in patients with GO.

In spite of their capacity to recognize retroorbital fibroblasts, retrobulbar T cells may fail to exert target cell cytotoxicity. When screened for their capacity to kill autologous retroorbital fibroblasts, five of the six lines failed to exert target cell

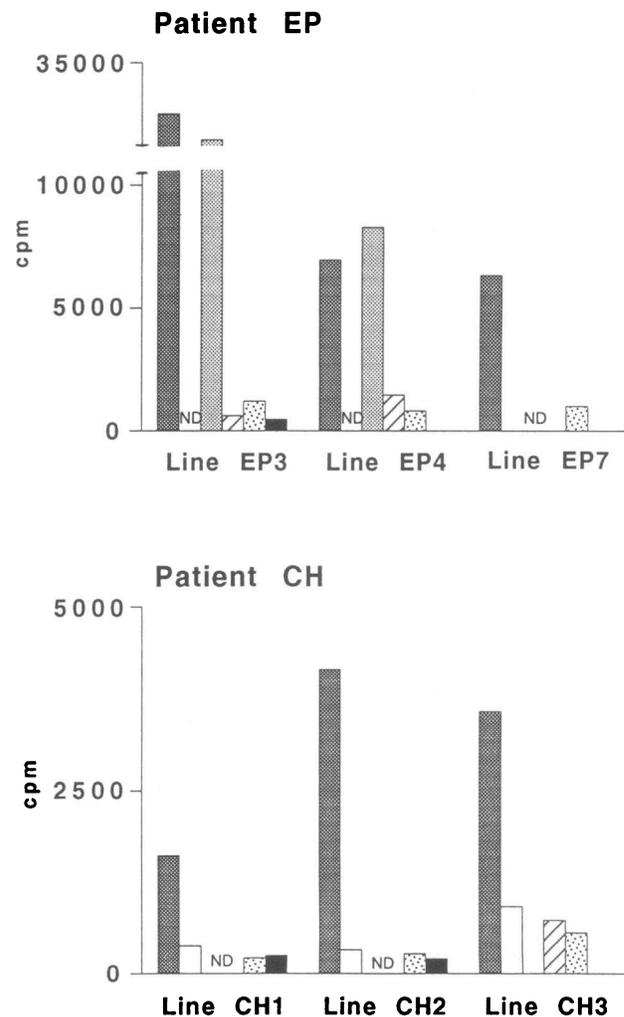


Figure 1. Retrobulbar T cell lines from two patients with GO specifically recognize autologous fibroblasts. Proliferation (³H]thymidine incorporation) of T cell lines in response to stimulation with irradiated autologous retrobulbar fibroblasts (■), irradiated autologous retrobulbar fibroblasts incubated with a blocking antibody against HLA class I (□), homogenized fibroblasts in combination with irradiated autologous PBMC (▨), irradiated autologous PBMC alone (⊖) or in combination with homogenized eye muscle fibers (▩). Results are expressed as mean counts per minute for triplicate cultures; variations among triplicate cultures were < 10%.

Table III. Retrobulbar T Cell Lines Recognize Autologous Fibroblasts

| Stimulus | Line 306CH1 | Line 306CH2 | Line 306CH3 |
|-------------------------|-------------|-------------|-------------|
| 0 | 451 | 112 | 562 |
| Retrobulbar fibroblasts | 2621 | 3240 | 3764 |
| Skin fibroblasts | 1237 | 1458 | 1970 |

Proliferation (^3H thymidine incorporation) of T cell lines in response to stimulation with irradiated retrobulbar fibroblasts and irradiated skin fibroblasts. Results are expressed as mean counts per minute for triplicate cultures; variations among triplicate cultures were <10%.

cytotoxicity (Fig. 2). Only one line gave a good cytotoxic response against autologous but not against allogeneic fibroblasts. This line had proliferated particularly well upon stimulation with autologous fibroblasts (Fig. 1). Our finding may thus indicate that target cell cytotoxicity of retrobulbar T cells depends on their antigen recognition potential. Some fibroblast-specific T cell populations may recognize their antigen and proliferate, but need not necessarily be capable of killing.

Discussion

We have generated and expanded T cell lines from the activated T cells present in the retrobulbar tissue from two patients with severe GO to study in detail their phenotypic and functional properties. All lines were $\text{CD8}^+\text{CD45RO}^+$, which was presumably due to a predominance of CD8^+ memory cells in the retrobulbar tissue of patients with GO, as reported previously (4, 21). This is of interest as the lymphocytic infiltrate in the Graves' disease thyroid consists primarily of CD4^+ cells (26), and uncloned T cell lines derived from Graves' disease thyroid tissue are predominantly CD4^+ (19).

The great majority of CD8^+ T cell clones from normal individuals, as well as from patients with GO, has been shown to possess a Th1-like cytokine secretion pattern, producing $\text{IFN-}\gamma$ and TNF, but not IL-4 (21, 27). Therefore, it seemed of interest to determine whether this was also true for our CD8^+ cell lines. They did, however, secrete $\text{IFN-}\gamma$ as well as IL-4.

Substantial IL-4 production by CD8^+ cells is a rare finding (28), but has been shown to be a feature of CD8^+ suppressor T cell clones (29). Suppressor functions may thus have been operative in our cell lines. In this context, it seems of special interest that retrobulbar T cell lines also secreted considerable amounts of IL-10. IL-10 has been described to be a general suppressor of immune responses (30). It has for instance been demonstrated to inhibit the production of $\text{TNF-}\alpha$ and $\text{TNF-}\beta$ (31) and to decrease the number of peptide MHC complexes available to the T cell receptor allowing only suboptimal T cell activation (32). Some of the typical functions of Th-1 cells may thus be attenuated or lost in CD8^+ T cell populations capable of simultaneous IL-10, IL-4, and $\text{IFN-}\gamma$ production. Loss of cytotoxic effector function could explain why five of six retrobulbar T cell lines failed to exert target cell cytotoxicity in spite of significant proliferation in response to antigenic stimulation. This observation is in good agreement with *in vivo* findings, which demonstrate fibroblast proliferation with no indication of target cell damage in spite of the presence of CD8^+ cells in the retrobulbar tissue from patients with GO (4, 5, 7, 9). Fibroblast activation and consecutive collagen and glycosaminoglycan production may be due to cytokines such as $\text{IFN-}\gamma$ secreted in the course of T cell activation (4, 33). Eye muscle enlargement, which is primarily the result of interstitial oedema and various intercellular deposits (6-9), may then be the consequence.

Obviously, it would be of great interest to know by which fibroblast antigen(s) T lymphocytes are attracted in the retrobulbar tissue. Our observation that retrobulbar T cells also respond, although at a lower level, to stimulation with skin fibroblasts suggests that the antigen in question is not unique to the orbita. However, its expression/presentation seems to be increased at the site of the disease. The reason for this overexpression/presentation is not yet clear. Viral infection has been shown to stimulate the metabolic functions of fibroblasts (34), and retroviruses have been mentioned frequently in the context of thyroid autoimmunity (35-38). Thus, it is tempting to speculate that viral infection may also be of importance in endocrine ophthalmopathy. Recent data from our laboratory demonstrate that antibodies against the *gag 2* protein of the human foamy virus bind to Graves' disease thyroids as well as to retrobulbar fibroblasts from patients with GO (39, 40). Future studies will have to demonstrate whether these findings are of pathogenetic significance.

Table IV. Retrobulbar T Cell Lines Do Not Derive from PBMC

| Stimulus | PBMC EP | PBMC CH | Line EP 4 | Line CH 2 | Line CH 3 |
|------------------------|---------|---------|-----------|-----------|-----------|
| 0 | 1446 | 193 | 1227 | 222 | 280 |
| Autologous fibroblasts | 1708 | 740 | 5113 | 4165 | 3585 |
| PPD | 7411 | 4803 | 1550 | 194 | 194 |
| Allogeneic PBMC | 21043 | 7761 | 1446 | 427 | 310 |

Proliferation (^3H thymidine incorporation) of PBMC and of retrobulbar T cell lines from two patients with GO in response to stimulation with irradiated autologous fibroblasts, PPD (100 $\mu\text{g}/\text{ml}$) in combination with irradiated autologous PBMC as antigen-presenting cells, and irradiated allogeneic PBMC. Results are expressed as mean counts per minute for triplicate cultures; variations among triplicate cultures were <10%.

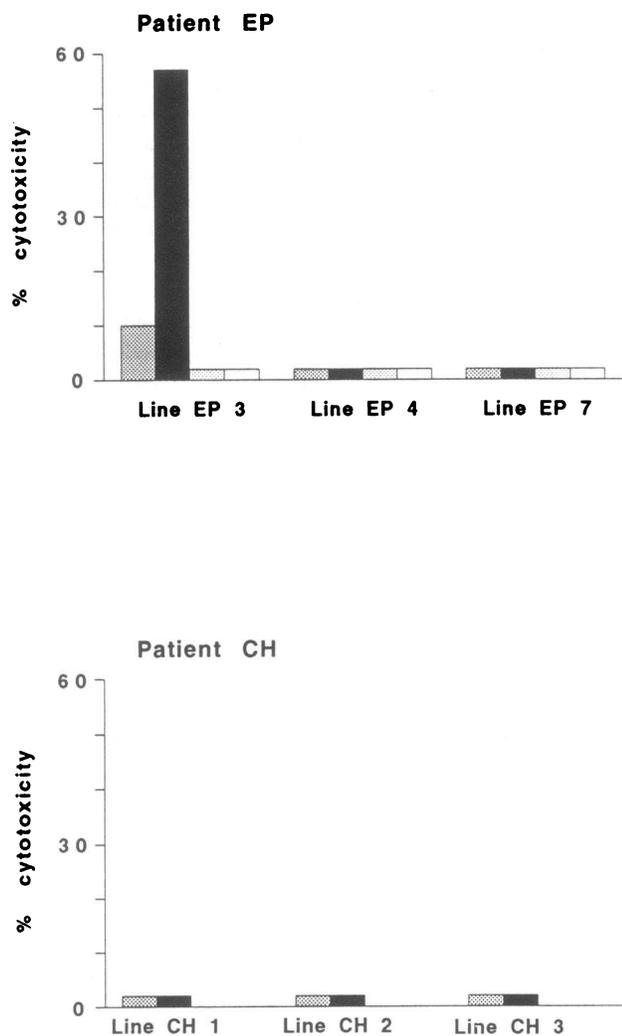


Figure 2. Fibroblast-specific retrobulbar T cell lines from two patients with GO may fail to exert target cell cytotoxicity. Target cells were chromium-51 labeled autologous or allogeneic retrobulbar fibroblasts. The target cell/effector cell ratios were 1:10 (■, autologous; ▣, allogeneic target cells) and 1:100 (■, autologous; □, allogeneic target cells). The results are expressed as percent lysis (experimental chromium-51 release minus spontaneous release divided by maximal release minus spontaneous release). Maximal chromium-51 release ranged between 18,500 and 20,000 CPM; spontaneous release was always < 20%.

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