TWO INDEPENDENT RECEPTORS ALLOW SELECTIVE TARGET LYSIS BY T CELL CLONES*

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Natural killer (NK)[1] cells are endowed with "spontaneous" cytolytic activity. They can be recovered from normal individuals and express an ability to lyse certain types of target cells (1, 2). When obtained under normal conditions, NK cells can be shown to represent a cellular subset with typical morphological and surface markers distinguishing them from any other classical cell type (3–5). NK cells have attracted great attention due to their ability to preferentially lyse malignant, compared with normal, cells of the same histogenetic origin (6, 7). Furthermore, NK cells display an ability to react against more primitive cells within the body, notably bone marrow stem cells (6) and can function as a major resistance barrier against bone marrow grafts (8).

When using activated cell populations in vitro, the situation becomes complicated and controversial. It has been suggested that, as measured by target cell specificity in cytolysis, monocytic (9) as well as T cell lines display NK activity. Cytolytic T cell lines have also been reported to lose their antigen-specific lytic ability while at the same time acquiring NK-like activity (10, 11). In other situations cell lines that behaved like NK cells have been obtained, but had partially different specificity profiles (11). Arguments have also been put forward advocating that NK cells may or may not express clonally derived receptors with varying specificities (12, 13).

We believe that our present results together with those obtained from binding studies by another group (14) can solve this by demonstrating the presence, in activated cells, of two receptors with different specificities for target killing.

Materials and Methods

Rats. DA rats ($RT1^a$), BN rats ($RT1^n$), and L. BN rats ($RT1^n$) were bred and maintained in our own colony.

Tumors. P1 and P2 sarcomas were induced in DA rats using dimethylbenzanthracene (DMBA) as described (15). Tumors were maintained in vivo by injecting single-cell suspensions subcutaneously into DA rats or alternatively grown in vitro in RPMI 1640 complemented with 10% fetal calf serum (FCS). P1-clones 9 and 10 were established by limiting dilution and selected for their susceptibility or resistance for P1-specific cytotoxic T cells as described (16). The PM tumor represents a spontaneous sarcoma from L.BN rats kept in vitro and in vivo in

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[1] *Abbreviations used in this paper:* CML, cell-mediated lysis; CTL, cytotoxic T lymphocytes; DMBA, dimethylbenzanthracene; EHAA, Eagles-high amino acid; FCS, fetal calf serum; 2-ME, 2-mercaptoethanol; MHC, major histocompatibility complex; NK, natural killer; PBS, phosphate-buffered saline; TCGF, T cell growth factor.

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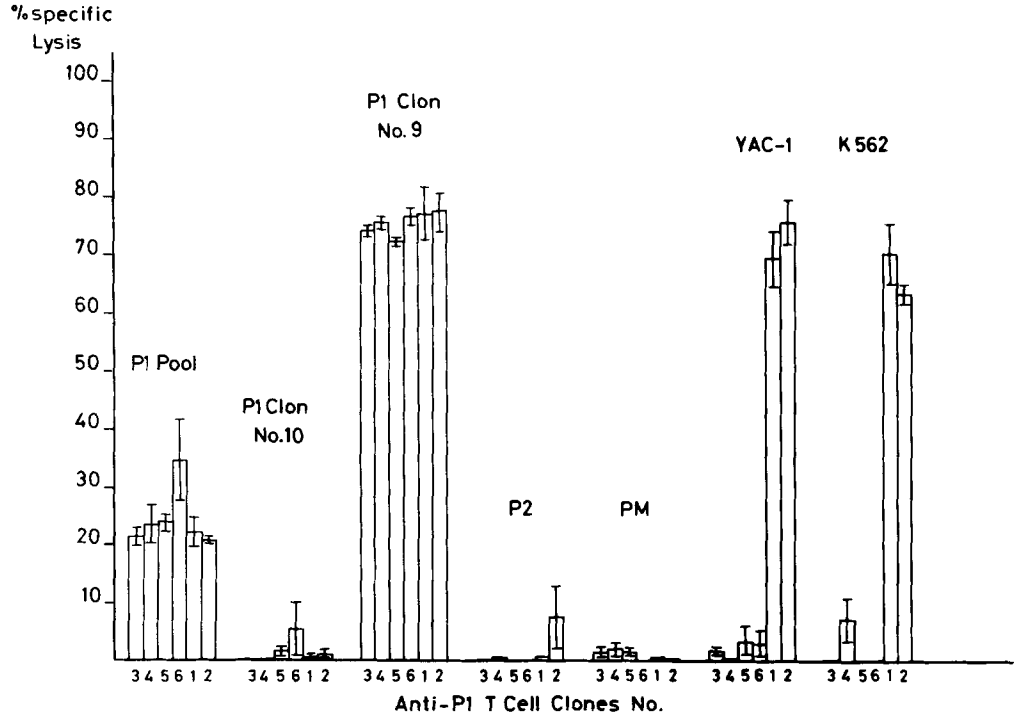

FIG. 1. P1 and P2 are DMBA induced sarcomas in DA rats. PM represents a spontaneous sarcoma in L.BN rats. P1 tumor cells were cloned and recloned by limiting dilution. Tumor cells from P1 clone 9 are susceptible and tumor cells from P1 clone 10 are resistant to lysis by DA anti-P1-specific killer T cells (16). Such cytolytic T cells clones are established as described under Material and Methods. Six different clones (1–6) were used for CML as described in Material and Methods using an effector/target cell ratio of 5:1.

syngeneic animals as described for P1 and P2. The characteristics of the tumor cell lines have been described elsewhere (15, 16). The human cell line K562 and mouse lymphoma YAC-1 were kept in vitro using Iscove's medium complemented with 10% FCS.

Preparation of Lymphoid Cell Suspensions and Rat T Lymphocytes. Rats were killed over carbon dry ice. Spleen and lymph nodes were aseptically removed. Single-cell suspensions were prepared in phosphate-buffered saline (PBS) (Ca^{++} and Mg^{++} free) using a stainless steel mesh. Erythrocytes were lysed by hypotonic shock using 0.9 ml of distilled water for 2 s followed by 0.1 ml of 10-times concentrated PBS. T lymphocytes were purified on 1.5 × 10 cm Ig anti-Ig columns (17) using sterile glass beads 80–120 mesh (Serva, Heidelberg, Federal Republic of Germany).

Preparation of T Cell Growth Factor (TCGF; Concanavalin A Supernatant). Rat spleen cells were cultured in Eagles high amino acid (EHAA) medium (18) complemented with 0.5% fresh normal BN rat serum and 5×10^{-5} M 2-mercaptoethanol (2-ME) and stimulated with 5 μg/ml of concanavalin A (Pharmacia Fine Chemicals, Uppsala, Sweden). 40 ml of a suspension containing 5×10^6 cells per ml were cultured in a 3024 Falcon tissue tissue culture flask (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA), and the supernatant was harvested 48 h later.

Restimulation of Primed DA T Lymphocytes with Antiidiotypic Antibodies. DA rats were immunized by six subcutaneous injections of 2×10^7 12,500-rad irradiated P1 sarcoma cells at 2-wk intervals. T lymphocytes from primed rats were stimulated with antiidiotypic antibodies in 3013 Falcon tissue culture flasks using EHAA medium complemented with 5×10^{-5} M 2-ME and 0.5% fresh normal BN rat serum as described (15). Antiidiotypic antiserum was used at

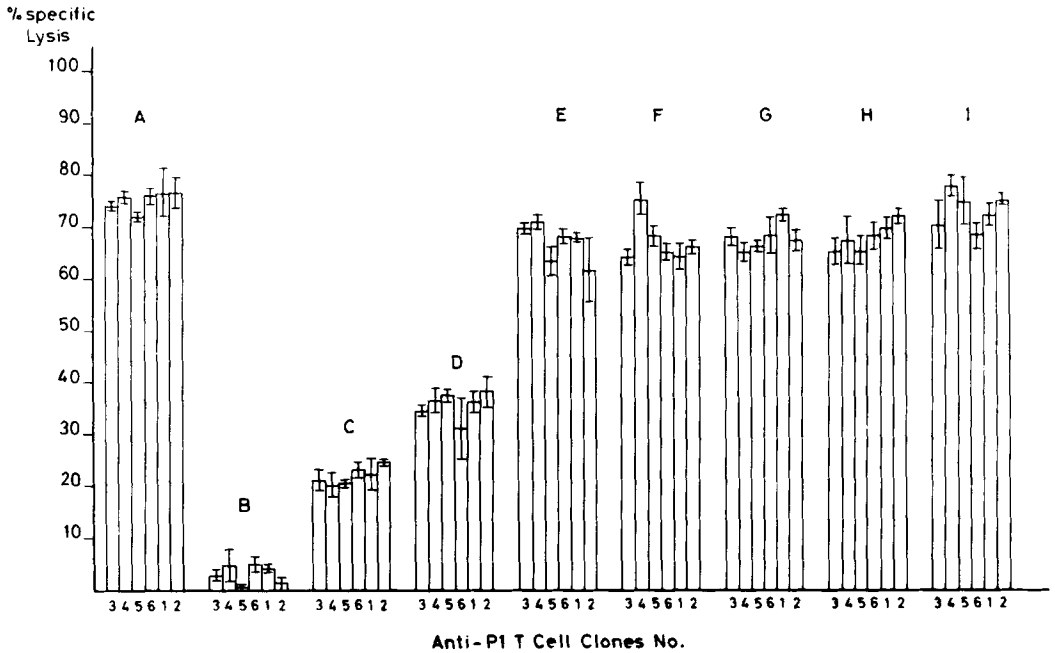

FIG. 2. DA anti-P cytolytic T cell clones 1-6 were confronted with ^{51}Cr-labeled target cells of P1 clone 9 (A) at an effector/target cell ratio of 5:1. Unlabeled tumor cells from P1 clone 9 were used for cold target inhibition at ratios of cold/hot target of 100:1 (B), 50:1 (C), 25:1 (D), and 1:1 (E). The same type of experiment was performed using unlabeled cells from P1 clone 10 at ratios of 100:1 (F), 50:1 (G), 25:1 (H), and 1:1 (I).

concentrations of 0.2%. Each flask contained 15 ml of a suspension containing 1.25×10^6 cell/ml.

Cloning of P1-specific Cytotoxic T Cells. In vitro restimulated DA T lymphocytes (see above) were collected on day 6 of culture and purified over Ficoll-Paque (19). Cells were expanded on DA macrophages and TCGF in the following way: Macrophages were harvested from the peritoneal cavity of DA rats, washed once, and irradiated with 2,500 rad. Macrophages obtained from one rat were distributed over three 3013 tissue culture flasks and cultured in 5 ml EHAA-medium complemented with 10% FCS, 5×10^{-5} M 2-ME, and 20% TCGF for 24 h. 10 ml of a suspension containing 1.25×10^6 purified restimulated T cells (see above) in EHAA medium complemented with 10% FCS, 20% TCGF, 5×10^{-5} M 2-ME, and 0.05% antiidiotypic antiserum was added to each flask. Cells were harvested 1 wk later and cloned and recloned on syngeneic macrophages by limiting dilution (16). Cytotoxic clones were selected and expanded as described above.

Cell-mediated Cytotoxicity Assay (CML). CML was performed in round-bottomed microtiter plates (Cook M24 ART; Greiner, Nuertingen, Germany) in 200 μl EHAA medium complemented with 5×10^{-5} M 2-ME and 5% heat-inactivated FCS. Assays were performed in triplicates with effector/target cell ratios as indicated in Figures using 10^4 ^{51}Cr-labeled target cells. The latter were prepared as described previously (16). Plates were incubated for 6 h at 37°C in 5% CO_2 in air. 100 μl of the supernatant was counted in the gamma counter. Percent cytotoxicity is expressed as 100 × [(experimental − spontaneous ^{51}Cr release)/(maximal − spontaneous ^{51}Cr release)].

Preparation of Antiidiotypic Antisera in DA Rats. DA anti-P1-specific antibodies were purified on paraformaldehyde-fixed P1 sarcoma cells as described previously (15). 4 mg of purified anti-P1 antibody was cross-linked with glutaraldehyde and injected together with complete Freund's adjuvant into 10 DA rats as described (20). The antiidiotypic nature of the harvested sera was

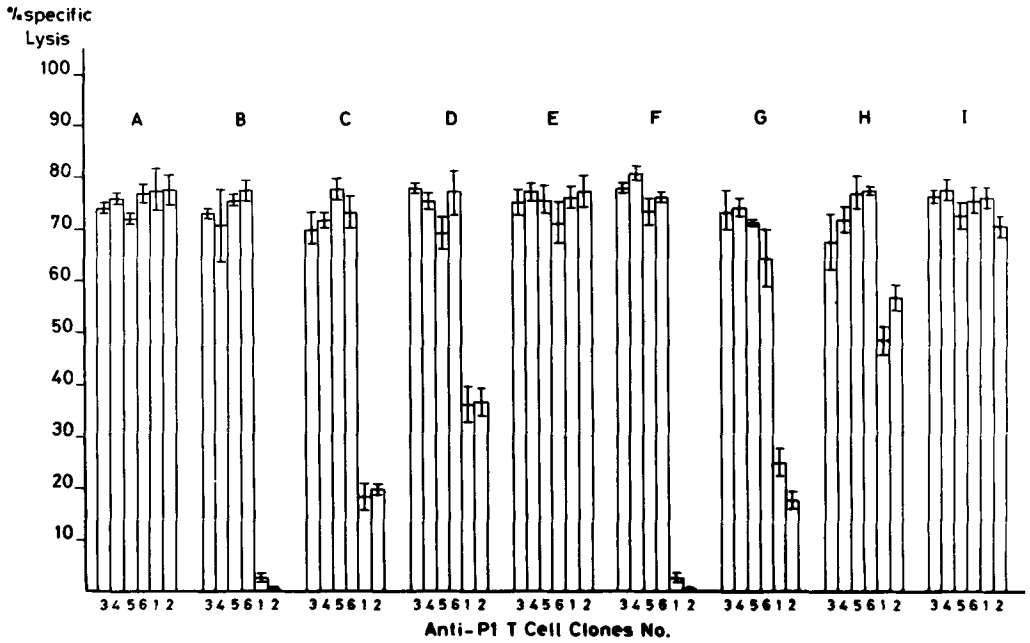

FIG. 3. Same type of experiment as described in the legend to Fig. 2. T cell clones 1–6 were tested against ^{51}Cr-labeled tumor cells from P1 clone 9 (A). Unlabeled K562 tumor cells were used for cold target inhibition at ratios of cold/hot target of 100:1 (B), 50:1 (C), 25:1 (D), and 1:1 (E). YAC-1 cells were used for cold target inhibition at ratios of 100:1 (F), 50:1 (G), 25:1 (H), and 1:1 (I).

determined by a solid-phase radioimmunoassay or by the restimulation assay using P1-primed DA T lymphocytes (15).

Results and Discussion

Our system involves the use of DMBA-induced sarcomas in DA rats, which allows the induction of tumor-specific T cell clones as well as production of corresponding auto-antiidiotypic antibodies (15). Such antibodies used at low concentration in the presence of TCGF can be used to selectively maintain specific T cell clones in vitro. In this system we have isolated six different T cell clones, all with idiotypic receptors and specific lytic ability for the relevant tumor (see Fig. 1). Two of these six clones, in addition, display a lytic profile identical to rat NK cells (see Fig. 1). This NK-like activity of clones 1 and 2 developed gradually during in vitro culture (16) and was discovered by specificity controls. Using cold target inhibition in a crisscross manner, we tested whether the very same cells in clones 1 and 2 display cytotoxic T lymphocyte (CTL) as well as NK specificity. Data shown in Figs. 2, 3, and 4 demonstrate that this is true. Two NK targets, YAC-1 and K562, block clone 1 and 2 (but not clone 3–6) effector cells from lysing the T cell-sensitive DA tumor P1-clone 9. It is of importance that P1-clone 9 target cells are resistant to normal splenic NK cells (see Fig. 5). In complete correspondence, P1-clone 9 targets could block the ability of T killer cell clones 1 and 2 to lyse either YAC-1 or K562 target cells (see Fig. 4).

It was crucial to establish whether clone 1 and 2 effector cells were using the same receptors to recognize the tumor-specific antigens on P1-clone 9 targets as those used to bind to YAC-1 or K562 cells. To answer this question we used antiidiotypic

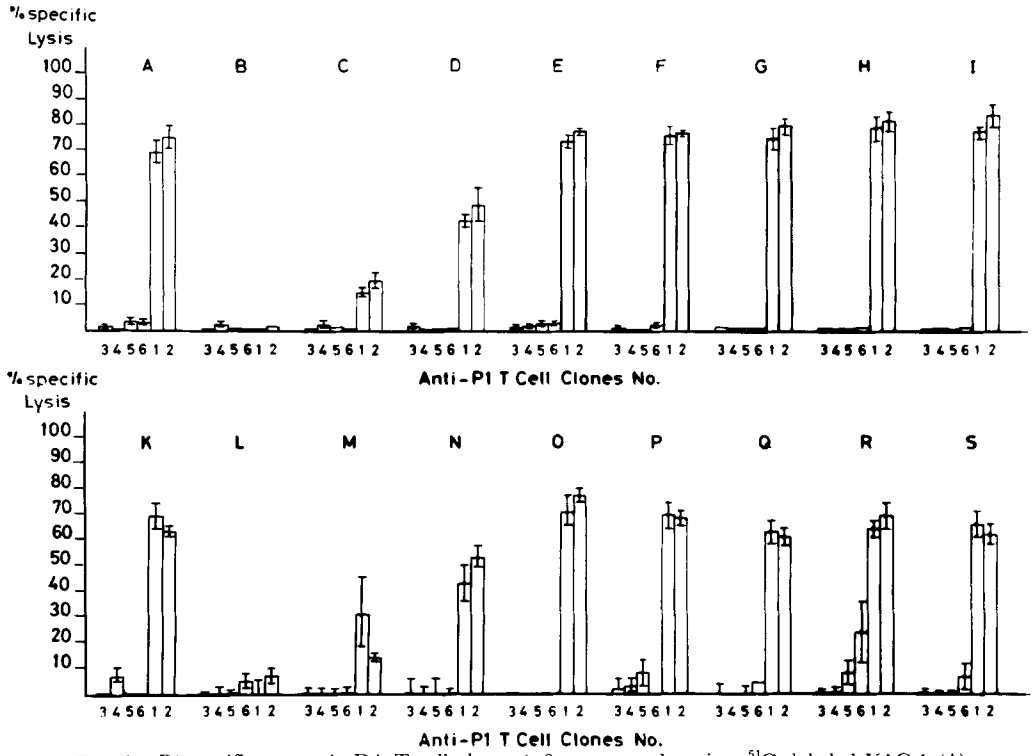

FIG. 4. P1-specific cytotoxic DA T cell clones 1–6 were tested against ^{51}Cr-labeled YAC-1 (A) (upper) and K562 (K) (lower) tumor cells. Tumor cells from P1 clones 9 and 10 were used for cold target inhibition. Unlabeled tumor cells from P1 clone 9 were used at ratios of 100:1 (B, L), 50:1 (C, M), 25:1 (D, N), and 1:1 (E, O). The same type of experiment at the same ratios was performed using cold targets from P1 clone 10. 100:1 (F, P), 50:1 (G, Q), 25:1 (H, R), and 1:1 (I, S).

antibodies in the presence or absence of complement (Fig. 6). Such antibodies in the presence of complement did wipe out all cytolytic activity in the clones regardless of the target type analyzed. However, at high concentration and in the absence of complement, a clear-cut selectivity of inhibition as to target type became apparent. Thus, clones 1 and 2 became inhibited in their reactions to P1 clone 9 targets (like the other clones), but were not suppressed at all in their reactivity towards the NK targets YAC-1 and K562. This strongly suggests the simultaneous presence of two distinct physically independent receptor types on clone 1 and 2 killer T cells: one that is idiotypic and antigen specific, and another displaying the binding profile of NK cells.

Our results demonstrate the possibility of the simultaneous expression of two distinct lytic systems with different specificities in the same T cell. Such parallel display may of course yield combined specificity profiles endowing the individual cell with a selectivity distinct from most normal CTL or NK cells. This could thus well explain existing controversies as to the specificities encountered when using immune-activated effector cells (21).

A second note of interest is the ability of these "double-expressing" T cells to lyse target cells (K562) devoid of major histocompatibility complex (MHC) antigens. Conventional cytolytic T cells normally fail to kill MHC-lacking targets, e.g., terato-carcinoma cells (22) and an NK-like receptor may be necessary here to allow such

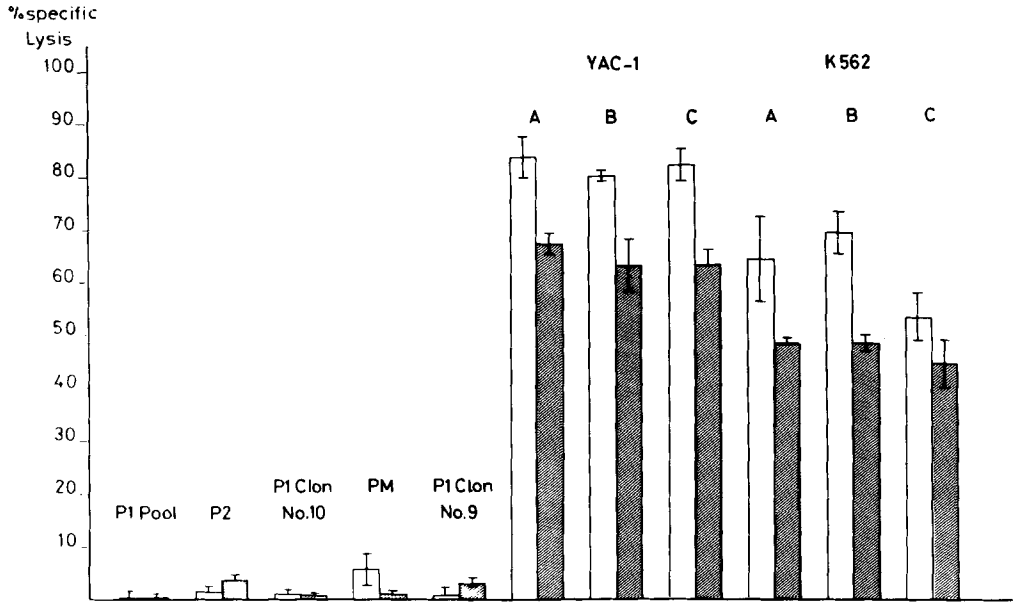

FIG. 5. Spleen cells from DA rats were used as a source of NK cells. They were confronted for 6 h with ^{51}Cr-labeled P1 and P2 sarcoma cells and cells from P1 clones 9 and 10 as well as with PM tumor cells at effector/target cell ratios of 100:1 (open columns) and 50:1 (▨). NK cells were also tested against the classical NK targets YAC-1 (A) and K562 (A) under the same conditions. Lytic capacity against YAC-1 and K562 targets was also tested in the presence of 3% antiidiotypic antiserum of specificity DA anti-(DA anti-P1) (15, 16) (B) or 3% DA normal serum (C).

lysis to occur. Whether such a receptor will indeed act as a triggering unit for the induction of a potential lytic machinery, like the IgG antibodies in antibody-dependent cellular cytotoxicity systems (23), is an important issue. Recent studies (14) have indicated that the ability to express an NK-like binding specificity is a feature that can be found on several types of bone marrow-derived cells. Our findings should thus not be considered as evidence for the inclusion of the conventional NK cells within the T cell lineage. The combination of lytic ability and NK binding specificity may, therefore, in a normal situation, be something uniquely restricted to the distinct cell type classically defined as an NK cell. Situations leading to the induction of lytic capacities in normally nonlytic cells may thus create an NK-like activity in non-NK effector cell types. Depending on the frequency of cells expressing NK-binding activity in such populations, one should expect the appearance, upon activation, of cells with dual specificities in the manner reported in this study.

Summary

Dimethylbenzanthracene-induced P1 sarcoma cells induce P1-specific antibodies in syngeneic DA rats. Antiidiotypic antibodies of specificity DA anti-(DA anti-P1) were induced against the tumor-specific antibodies and used to restimulate P1-primed DA T cells in vitro. Using antiidiotypic antibodies and T cell growth factor, P1-specific cytotoxic DA T cell clones were established by limiting dilution and kept in vitro. Two of these clones acquired during culture periods in addition to the P1 specificity lytic activity towards natural killer (NK) targets YAC-1 or K562. Cold

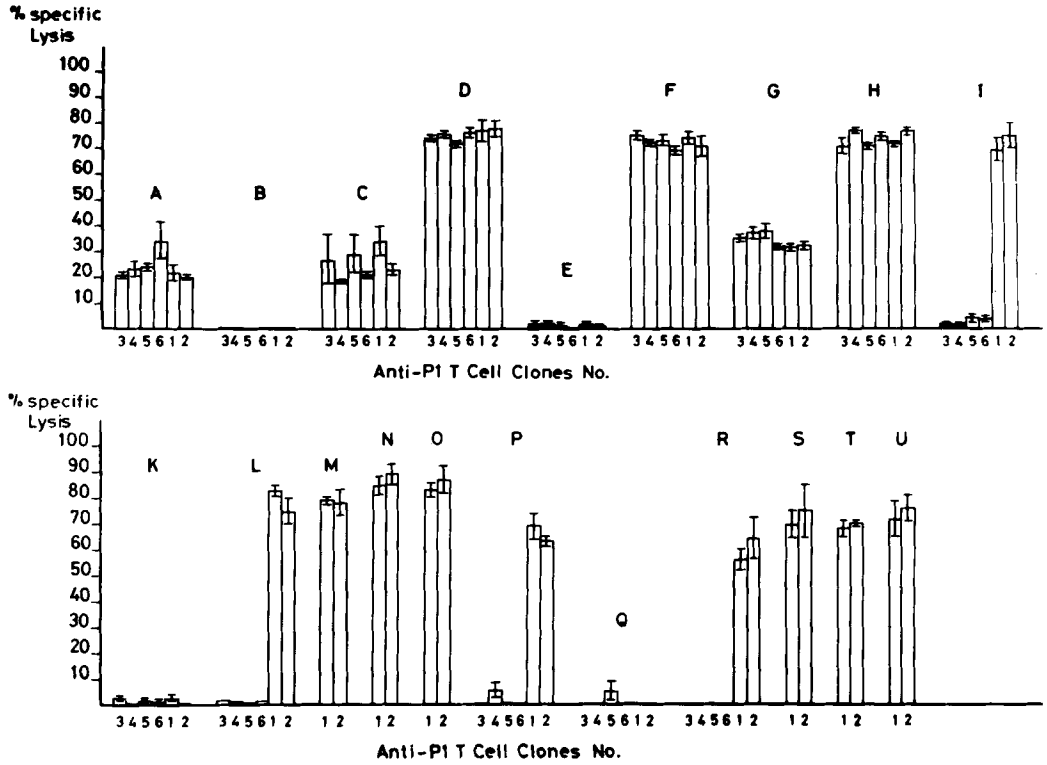

FIG. 6. DA anti-P1 cytotoxic T cell clones (1-6) were used as effector cells at an effector/target cell ratio of 5:1. (A) Targets, P1 sarcoma cells; effector cell untreated; (B) Targets, P1 sarcoma cells; effector cells treated with 1:10 diluted DA anti-(DA anti-P1) antiidiotypic antiserum and complement (21). (C) Targets, P1 sarcoma cells; effector cells treated with 1:10 diluted normal DA serum and complement. (D) Targets, tumor cells from P1 clone 9; effector cells untreated. (E) Targets, tumor cells from P1 clone 9; effector cells treated with antiidiotypic antiserum and complement (see B). (F) Targets, tumor cells from P1 clone 9; effector cells treated with normal DA serum and complement. (G) Targets, tumor cells from P1 clone 9; 3% of antiidiotypic antiserum was present during test period. (H) Targets, tumor cells from P1 clone 9; 3% of antiidiotypic antiserum absorbed on P1 specific DA T cell clones 1-6 was present during test period. (I) Targets, YAC-1; effector cells untreated. (K) Targets, YAC-1; effector cells treated with antiidiotypic antiserum and complement. (L) Targets, YAC-1; effector cells treated with normal DA serum and complement. (M) Targets, YAC-1; 3% of antiidiotypic antiserum was present during test period. (N) Targets, YAC-1; 3% absorbed (see H) antiidiotypic antiserum was present during test period. (O) Targets, YAC-1; 3% normal DA serum was present during test period. (P) Targets, K562; effector cells untreated. (Q) Targets, K562; effector cells treated with antiidiotypic antiserum and complement. (R) Targets, K562; effector cells treated with DA normal serum and complement. (S) Targets, K562; 3% of antiidiotypic antiserum was present during test period. (T) Targets, K562; 3% of absorbed antiidiotypic antiserum was present during test period. (U) Targets, K562; 3% of normal DA serum was present during test period.

target inhibition experiments showed that the very same cytotoxic T cells kill P1 and NK targets. Antiidiotypic antibodies of specificity DA anti-(DA anti-P1) inhibited cytotoxicity against P1 but not against YAC-1 or K562. We conclude that two independent receptors are located on these double-reactive T cell clones, one that is idiotypic and antigen-specific, and another displaying the binding profile of NK cells.

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