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LFA-1 but not Lyt-2 is associated with killing activity of cytotoxic T lymphocyte hybridomas

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The mechanism by which cytotoxic T lymphocytes (CTL) recognize and lyse target cells is largely unknown. Recently, two lymphocyte cell-surface glycoproteins, Lyt-2[1-4] and LFA-1 (lymphocyte function-associated antigen)[5-8], have been implicated in the killing activity of murine CTL. This suggestion was based mainly on the unique ability of antibodies directed against Lyt-2[1-4] and LFA-1[5-8] to inhibit cytotoxicity of CTL by affecting the killer cell. Lyt-2, a glycoprotein composed of subunits of molecular weights of 30,000–35,000 (30–35K), has been proposed as a common marker for CTL[9], essential for specific recognition of target cells[10]. This view, however, is incompatible with other results[11-14]. The newly detected LFA-1 molecule, containing subunits of 95K and 180K[5-8,15], is associated with the ability of CTL to bind target cells[16,17]. Most analyses of CTL structure–function relationships are ambiguous since they have been obtained with heterogeneous CTL populations. We have recently developed CTL-hybridomas which lyse specifically allogeneic tumour cells *in vitro*[18] and grow independently of exogenous stimuli[19]. In this study we have tested the relevance of Lyt-2 and LFA-1 antigens to cytotoxicity by analysing the CTL-hybridomas with a panel of anti-LFA-1 and anti-Lyt-2 monoclonal antibodies. We show that two monoclonal CTL-hybridomas have the phenotype LFA-1⁺ and Lyt-2⁻ and that their killing activity is inhibited by anti-LFA-1 but not by anti-Lyt-2. The results suggest that LFA-1 is involved in CTL-cytotoxicity, while Lyt-2 molecules are not required.

CTL somatic hybrids, in which elimination of functionally irrelevant molecules could occur due to chromosomal segregation or to repression controlled by the tumour cell parent, provide a new homogeneous and antigen-specific source for studies of specific effector structures participating in cytotoxicity. We analysed the effect of various monoclonal antibodies on two unrelated CTL-hybrid subclones, Md. 26 and P. 47, that had been derived from BALB/c anti-EL4 ($H-2^d$ anti-$H-2^b$) CTL following secondary stimulation either in mixed lymphocyte culture (MLC) or *in vivo* (peritoneal exudate lymphocytes, PEL), respectively[18]. These monoclonal CTL-hybridomas retained their killing specificity towards $H-2^b$ tumour target cells during prolonged continuous growth in culture[20] and exhibited this capacity while the studies described here were carried out. The monoclonal antibodies examined had been raised against CTL and selected according to their ability to inhibit killing by binding to the effector cells[5,7,17,21].

The presence of the differentiation antigens Lyt-1, Lyt-2 and LFA-1 on the CTL hybridomas and heterogeneous MLC population was examined by immunofluorescence staining. The analysis (Fig. 1*a*) demonstrates the presence of all three antigens on MLC, low levels of Lyt-1 on P but not on M hybridomas and high density of LFA-1 on both hybridomas. None of the CTL-hybridomas were stained with anti-Lyt-2. Additional monoclonal anti-Lyt-2 (H35-17.2[7,17], M12/4, M12/5 and M12/7.2[21]), failed to stain the hybridomas, while several anti-LFA-1 monoclonal antibodies (H35-89.9[7] and M17.5.2[5])

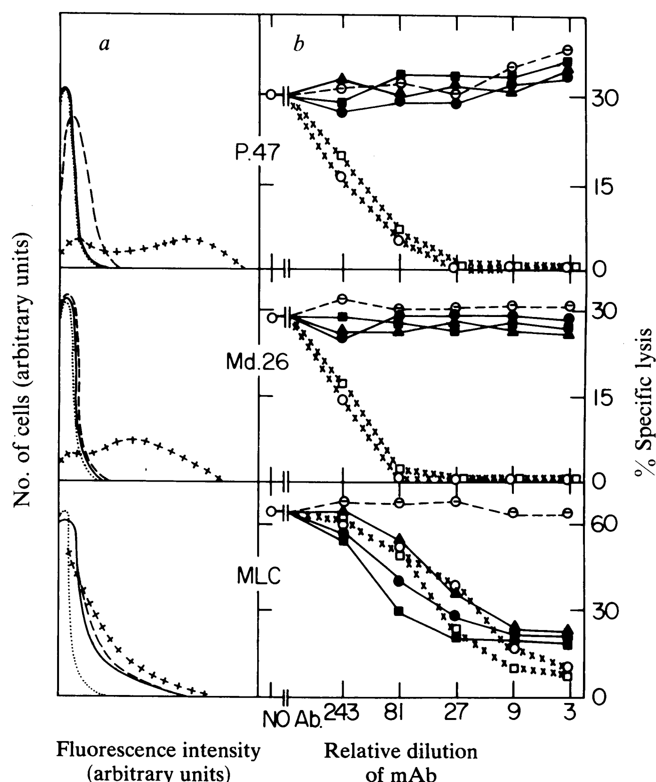

Fig. 1 Expression of Lyt and LFA-1 antigens on CTL-hybridomas and the effect of anti-Lyt and anti-LFA-1 mAb on cytotoxicity. *a*, Cytofluorometric analysis of Lyt-1, Lyt-2 and LFA-1 on CTL and CTL-hybridomas was done by reacting 2×10^6 MLC effector cells (primary BALB/c anti-C57BL/6 spleen cells stimulated for 5 days at 1:1 ratio) or CTL-hybridoma clones P.47 or Md.26 with the following monoclonal antibodies: anti-Lyt-1 (- - - -) and anti-Lyt-2 (——) produced by hybridomas 53.7.3 and 53.6.7, respectively[22] (these were obtained from the Cell Distribution Center of the Salk Institute, San Diego); anti-LFA-1 (+++) produced by hybridoma H35-89.9[7] and concentrated from ascites by 50% $(NH_4)_2SO_4$. Washed cells were stained with fluoresceinated rabbit anti-rat immunoglobin. Samples were analysed using a fluorescence-activated cell sorter (FACS II). For each cell preparation, a control staining with the fluoresceinated reagent alone (· · · ·) is shown. Data are expressed in arbitrary units as obtained from the cell sorter. *b*, The effect of anti-Lyt and anti-LFA-1 monoclonal antibodies on cytotoxicity of MLC and CTL hybridomas was studied by incubating 10^4 effector cells in microtitre plates with different dilutions of the following monoclonal antibodies: anti-Lyt-1, 53.7.3[22] five times concentrated (- - - - ○); anti-Lyt-2 were 53.6.7[22] five times concentrated (▲); H35-17.2[17] 1:3 diluted (●); and M12/7.2[5] 1:10 diluted (■); anti-LFA-1 were H35-89.9[7] at 1:1,000 dilution (+++○); and M17/5.2[5] at 1:30 dilution (+++□). The concentrations of antibodies listed above indicate the original stocks which were serially diluted in the assay. After 20 min incubation at 37 °C in 100 μl reaction medium (RPMI containing 10% fetal calf serum and 10 mM HEPES *p*H 7.4), 10^4 ^{51}Cr-labelled, neuraminidase-treated[18] EL4 target cells were added in 50 μl reaction medium. Plates were spun for 5 min at 500 r.p.m. and incubated for 5 h at 37 °C. Lysis was stopped by cooling, plates were spun for 10 min at 2,000 r.p.m. and 75 μl supernatants were taken to determine the ^{51}Cr release. Spontaneous release was 12%.

stained over 85% of the hybrid cells (not shown). The relevance of Lyt-2 and LFA-1 to the killing activity was further studied by testing the effect of the monoclonal antibodies on cytotoxicity (Fig. 1*b*). The hybridomas or BALB/c anti-C57BL/6 MLC were incubated with ^{51}Cr-labelled EL4 target cells in the presence of diluted antibody and specific killing was determined. As previously reported, cytotoxicity of MLC was inhibited by anti-Lyt-2[1-4] and by anti-LFA-1[5-8] and was unaffected by anti-

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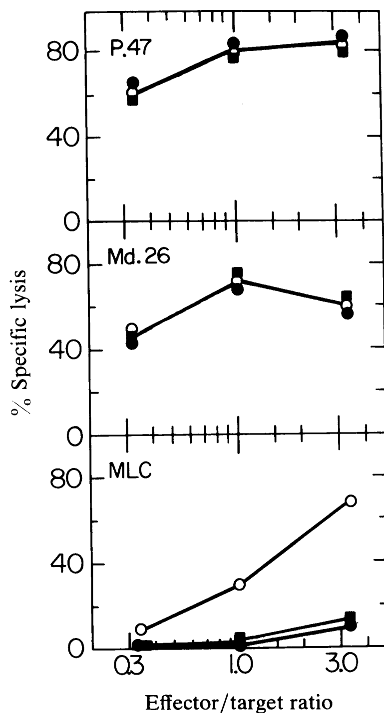

Fig. 2 The effect of pretreatments with anti-Lyt-2 and complement on the cytotoxicity of CTL-hybridomas and MLC. CTL-hybridomas and primary MLC (see Fig. 1 legend) were preincubated for 45 min at 37 °C with anti-Lyt-2; H35-17.2[17] (●) or M12.7.2[5] (■) and 1:10 dilution of fresh guinea pig serum as complement or with complement alone (○). After washing, treated cells were diluted and cytotoxicity was tested on 10^4 neuraminidase-treated ^{51}Cr-labelled EL4 target cells. E/T ratio was calculated according to number of effector cells before treatment. Incubation was for 5.5 h and spontaneous release was 18%.

Lyt-1. The killing activity of both CTL-hybridomas was likewise unaffected by anti-Lyt-1 and completely abolished by two anti-LFA-1 antibodies. However, unlike parental MLC, the cytotoxicity of the hybridomas was unaffected by three different anti-Lyt-2 monoclonal antibodies. The absence of detectable Lyt-2 on the surface of the CTL-hybridoma was further confirmed by preincubation of both M and P hybrid cells with two different anti-Lyt-2 antibodies in the presence of complement (Fig. 2). Such pretreatment completely eliminated the cytotoxicity of parental MLC but did not affect the cytolytic potential of hybridomas. The inability to detect Lyt-2 on the CTL hybridomas did not result from a wrong specificity or a low titre of the antibody used; since antibody 53.6, which recognizes a nonpolymorphic portion of Lyt-2 molecule[22] and two other monoclonal anti-Lyt-2 molecules, did bind to parental MLC killers and inhibited very effectively their cytotoxicity, while the same monoclonal antibodies could neither bind to the hybrid cells (Figs 1*a* and 2) nor affect their killing activity (Fig. 1*b*). Furthermore, more than a single Lyt-2 antigenic determinant was absent, since the hybridomas were unreactive with antibodies M12/4, M12/5 and M12/7, which define three topographically distinct Lyt-2 determinants (not shown).

In contrast to anti-Lyt-2, four different monoclonal anti-LFA-1 molecules were all equally effective in inhibiting specific killing of EL4 and ALB.B2 target cells by the hybridomas (Fig. 1*b* and not shown). Since LFA-1 molecules are expressed not only on the CTL-hybridomas but also on EL4 (ref. 15 and our unpublished results), which had been used as target cells in these experiments, it was necessary to examine whether anti-LFA-1 exerts its inhibitory effect on killing through its binding to the target cells or to the effector cells, as previously suggested[5–8]. This was tested by preincubating anti-LFA-1 separately with either isolated CTL-hybridomas or EL4 cells and combining treated and untreated components for the killing assay after excess of the antibody was removed. Cytotoxicity of preincubated CTL hybridomas was completely abolished by bound anti-LFA-1 molecules (Fig. 3), while pretreated EL4 target cells were unaffected, showing that indeed, anti-LFA-1 inhibited killing by affecting primarily the CTL-hybridomas rather than EL4 target cells.

The hypothesis that Lyt-2 is associated with the CTL receptor was based on a genetic linkage between Lyt-2-3 locus and the V_k locus[23], on the observation that anti-Lyt-2 inhibits killing at the recognition-adhesion stage[24], and on a mutagenized CTL Lyt-2^+ clone which lost in parallel Lyt-2 and the ability to kill specifically TC[10]. Recently, however, MacDonald *et al.*[14] reported that some CTL which express Lyt-2 are not inhibited by anti-Lyt-2. In addition, it becomes apparent that CTL populations[12] and long-terms lines[11,13] directed against Ia antigens (class II) major histocompatibility complex (MHC) are Lyt-$1^+2^\mp$ and helper cells specific for class I MHC antigens are Lyt-1^+2^+ and their helper function is inhibited by anti-Lyt-2[26]. These results prompted Swain[26] to suggest that Lyt phenotype correlates primarily with the class of MHC antigen recognized rather than with function (see also ref. 27 for a recent discussion). Our observation that functional CTL-hybridomas are Lyt-2 negative support the notion that Lyt-2 molecules are not required for specific cytotoxicity. The H-2 specificity of these CTL-hybridomas was recently mapped to the *D* region of $H\text{-}2^b$ by using target cells from recombinant mice[28] and by inhibition of the hybridoma-mediated target cell killing with anti-H-$2D^b$[19]. In this respect, the CTL-hybridomas seem to be exceptional, since all CTL directed against class I MHC were reported so far as Lyt-2^+. However, hybridomas are often subjected to selective loss of some parental chromosomes or to repression of specific genes, hence it is possible that the CTL

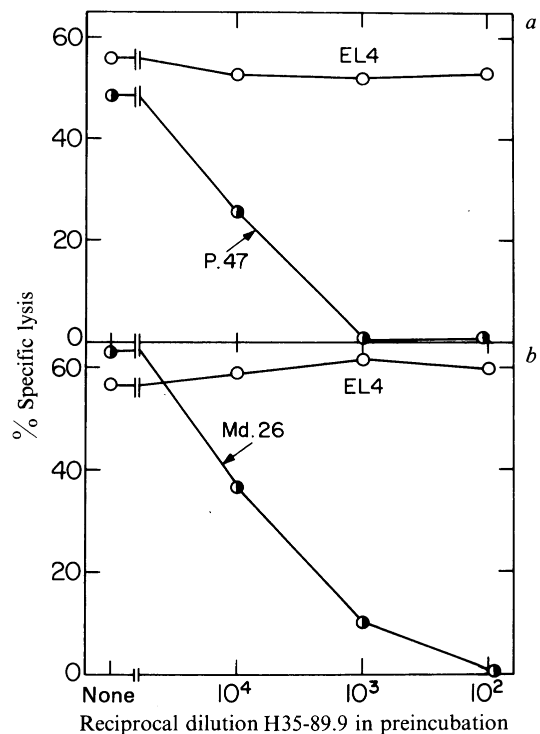

Fig. 3 Preincubation of CTL-hybridomas but not of EL4 target cells with monoclonal anti-LFA-1 inhibits cytotoxicity. Isolated CTL-hybridomas or neuraminidase-treated ^{51}Cr-labelled EL4 cells were incubated with anti-LFA-1 H35-89.9[7] at the indicated dilutions for 60 min at 37 °C. Cells were washed three times from excess of monoclonal antibody and cytolytic reactions were completed with an equivalent number of the missing untreated effector or target cells. Killing assays contained 10^4 CTL-hybridomas (either P.47 (*a*) or Md.26 (*b*)).

hybridomas analysed here originated from Lyt-2$^+$ parental CTL whose Lyt-2 expression was lost. Regardless of the history of these cells, the mere fact that they specifically lyse target cells without expressing Lyt-2 antigens strongly suggests that Lyt-2 molecules are not essential for either target cell recognition or binding. This is in contrast to previous suggestions that Lyt-2 is an antigen receptor. However, it is possible that Lyt-2 is a non-antigen-specific receptor that, together with the antigen receptor, contributes to the avidity of the CTL for the target cells[17,21]. In cases where the antigen receptor has low avidity, loss of the Lyt-2 interaction would result in loss of CTL activity, explaining the antibody inhibition[1–4] and Lyt-2 mutant results[10]. Thus, while in some cases Lyt-2 may contribute to CTL activity, we conclude that it is not essential and is distinct from the antigen receptor.

LFA-1 molecules, unlike Lyt-2, may be an essential structure of functional CTL. This conclusion is based on the observation that *all* CTL populations and clones examined so far express LFA-1 and their cytotoxicity is inhibited by anti-LFA-1[5-8]. The formation of effector target cell conjugates is inhibited by anti-LFA-1[16,17], indicating that the binding stage is affected. The ability of anti-LFA-1 to inhibit specific lysis irrespective of the TC specificity[7,16], as well as concanavalin A mediated nonspecific killing[7,17], emphasizes the general role of LFA-1 in a fruitful CTL target cell interaction. Previous findings that anti-LFA-1 interfere with other T-cell functions like proliferative MLC responses, macrophage-dependent antigen-specific proliferation, and T cell-dependent antibody responses to red cells[7,16,21], suggest that LFA-1 is crucial for the interaction of T cells with other cells. The fact that a panel of anti-LFA-1 monoclonal antibodies inhibited very efficiently the specific cytotoxicity of CTL-hybridomas (Figs 1*b*, 3) and immunoprecipitated from the hybrid cells the 95K and 180K LFA-1 subunits (not shown) indicates that the same LFA-1 molecules that participate in parental CTL cytotoxicity also participate in the specific target cell killing induced by the hybridomas. The monoclonal CTL hybridomas which grow rapidly in culture and retain specific killing activity for months without added stimuli, provide an unlimited and homogeneous source for studying the molecular basis for the role of LFA-1 in T cell-mediated cytotoxicity.

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1. Nakayama, E., Shiku, H., Stockert, E., Oettgen, H. F. & Old, L. J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1977–1981 (1979).
2. Shinohara, N. & Sachs, D. H. *J. exp. Med.* **150**, 432–444 (1979).
3. Hollander, N., Pillemer, E. & Weissman, I. *J. exp. Med.* **152**, 674–687 (1980).
4. Sarmiento, A., Glasebrook, A. L. & Fitch, F. W. *J. Immun.* **125**, 2665–2672 (1980).
5. Davignon, D., Martz, E., Reynolds, T., Kürzinger, K. & Springer, T. A. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4535–4539 (1981).
6. Kürzinger, K., Reynolds, T., Germain, R. N., Davignon, D., Martz, E. & Springer, T. A. *J. Immun.* **127**, 596–602 (1981).
7. Pierres, M., Goridis, C. & Golstein, P. *Eur. J. Immun.* **12**, 60–69 (1982).
8. Dialynas, D., Loken, M., Sarmiento, M. & Fitch, F. W. *Mechanisms of Cell-Mediated Cytotoxicity* (eds Clark, W. R. & Golstein, P.) 547–556 (Plenum, New York, 1982).
9. Cantor, H. & Boyse, E. A. *Cold Spring Harb. Symp. quant. Biol.* **41**, 23–31 (1977).
10. Dialynas, D. P., Loken, M. R., Glasebrook, A. L. & Fitch, F. W. *J. exp. Med.* **153**, 595–604 (1981).
11. Swain, S. L., Dennert, G., Wormsley, S. & Dutton, R. *Eur. J. Immun.* **11**, 175–180 (1981).
12. Vidovic, D., Juretic, A., Nagy, Z. A. & Klein, J. *Eur. J. Immun.* **11**, 499–504 (1981).
13. Pierres, A., Schmitt-Verhulst, A.-M., Buferne, M., Golstein, P. & Pierres, M. *Scand. J. Immun.* (in the press).
14. MacDonald, H. R., Thiernesse, N. & Cerottini, J. C. *J. Immun.* **126**, 1671–1675 (1981).
15. Kürzinger, K., Ho, M. K. & Springer, T. A. *Nature* **296**, 668–670 (1982).
16. Davignon, D., Martz, E., Reynolds, T., Kürzinger, K. & Springer, T. A. *J. Immun.* **127**, 590–595 (1981).
17. Golstein, P. *et al. Immun. Rev.* (in the press).
18. Kaufmann, Y., Berke, G. & Eshhar, Z. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2502–2506 (1981).
19. Eshhar, Z., Waks, T., Oren, T., Berke, G. & Kaufmann, Y. *Curr. Topics Microbiol. Immun.* **100**, 11–17 (1982).
20. Kaufmann, Y., Berke, G. & Eshhar, Z. *Lymphokines, Monoclonal, T Cells and Their Products* Vol. 5 (eds Feldman, M. & Schreier, M. H.) 277–289 (Academic, New York, 1982).
21. Springer, T. A., Davignon, D., Ho, M.-K., Kürzinger, K., Martz, E. & Sanchez-Madrid, F. *Immun. Rev.* **68** (in the press).
22. Ledbetter, J. A. & Herzenberg, L. A. *Immun. Rev.* **47**, 63–89 (1979).
23. Gibson, D. M., Taylor, B. A. & Cherry, M. *J. Immun.* **121**, 1585–1590 (1978).
24. Fan, J., Ahmed., A. & Bonavida, B. *J. Immun.* **125**, 2444–2453 (1980).
25. Swain, S. L. & Fanfili, P. R. *J. Immun.* **122**, 383–391 (1979).
26. Swain, S. L. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7101–7105 (1981).
27. Simpson, E. *Nature* **295**, 366–367 (1982).
28. Kaufmann, Y. *Transplantn Proc.* (in the press).