In Monoclonal antibodies and T cell hybridomas. U. Hammerling, G. Hammerling, and J. Kearney, editors. Elsevier, New York. 45-52. 1981.

1.2.4. Monoclonal antibodies as probes of surface structures participating in T-lymphocyte function

TIMOTHY A. SPRINGER, KONRAD KÜRZINGER, TIMOTHY REYNOLDS, RONALD N. GERMAIN, DENISE DAVIGNON and ERIC MARTZ

Laboratory of Membrane Immunochemistry, Sidney Farber Cancer Institute, Harvard Medical School, Boston; and Department of Microbiology, University of Massachusetts, Amherst (Massachusetts, U.S.A.)

Introduction

While much has been learned in the past decade about T-cell subsets and their effector functions, the structure and mechanism of action of the molecules which participate in specific immune cellular functions remain largely unknown. Antigen-specific cytolytic T lymphocytes (CTL) are assayed by their ability to kill target cells and provide one of the simplest systems for studying cell-mediated immunity. Only two cells are involved, the CTL effector and the target, and the endpoint of release of radioisotopically tagged molecules from lysed target cells can be measured after a short time period of 2–4 h. Nonetheless, CTL-mediated killing is a complicated, multistep process. It involves crawling to reach the target or surface motility during target engagement, specific antigen recognition, adhesion, lethal-hit triggering, and delivery of the lethal hit (reviewed in refs. 1–3). Therefore, it is likely that a number of different surface structures participate in the CTL functional pathway. It would be of great interest to isolate and study these molecules. Therefore, we have explored the use of monoclonal antibodies (MAb) as probes for surface molecules of critical importance in CTL-mediated lysis of tumor cells.

Methods

The work reviewed here is being published in a more detailed form elsewhere[4–6]. Briefly, spleen cells from rats immunized to mouse cytolytic T lymphocytes were fused to NSI myeloma cells. Using the indirect binding assay, hybrid cells secreting

antibodies binding preferentially to T lymphocytes were selected for cloning and further analysis. Methods were as previously described[7,8].

Results

A panel of MAb, almost all of which reacted with antigens expressed on the surfaces of activated T cells, was tested for the blocking of CTL function in the absence of complement (Fig. 1). The idea was that a MAb binding to a structure involved in CTL-mediated lysis should cause either steric hindrance or removal of the structure from the cell surface, thereby inhibiting killing. On the other hand, binding to a CTL surface structure which does not participate in the killing pathway should have no effect. To select MAb which blocked by binding to the effector cell rather than the target cell, a xenogeneic mouse CTL anti-rat tumor target cell system was utilized for screening (MAb and target cells from same species).

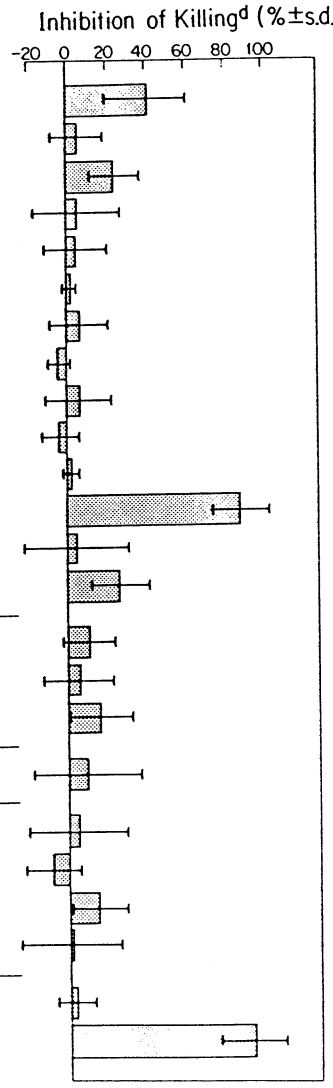
These experiments suggested that the majority of CTL surface antigens do not participate in killing. MAb to Thy-1, Ly 6, H-2, Lyt-1, Ly 5, an antigen apparently identical to Lgp100[9], and previously undescribed polypeptides of M_r = 115,000, 140,000 and 250,000, 60,000 and 46,000 had little or no effect on CTL function (Fig. 1). Antisera to some of these and other antigens have also previously been shown not to block (reviewed in ref. 1).

In contrast, two other antigens do appear to participate in CTL-mediated killing. Antibodies to the first antigen, the Lyt-2,3 complex, have previously been reported to block allogeneic CTL-mediated lysis[10–15], and we confirmed this in our xenogeneic system (Fig. 1). The 53.6 and M5/24 anti-Lyt-2,3 MAb gave 90 % and 40 % inhibition of killing, respectively (Fig. 1). We confirmed that the Lyt-2,3 antigen

---

Fig. 1. Effects of monoclonal antibodies on C57BL/6 anti-BN lymphoma CTL-mediated killing. Effector cells were pretreated with an equal volume of dialyzed hybridoma culture supernatant for 30 min at 20° C, then ^{51}Cr-labeled BN lymphoma cells were added, and the assay carried out as described previously[1]. Percent inhibition of corrected ^{51}Cr release is expressed relative to cultures treated with dialyzed NSI culture supernatant. MAb are grouped according to criteria used for isolation or their source. A) Selected for ratio > 4 of indirect binding to C57BL/6 Con A blasts vs. nu/nu BALB/c spleen; 2 clones later proved to be allospecific rather than T cell-specific. B) Immunoprecipitation of interesting ^{125}I-labelled polypeptides. C) Lysis of P815 and EL-4 tumors. D) Previously characterized MAb[8] reactive with both B and T cells. E) Lyt-1 and Lyt-2 MAb described by Ledbetter and Herzenberg[9]. a: Determined by double immunodiffusion as previously described[8]. b: Ratio of ^{125}I-labelled rabbit anti-rat IgG bound in the indirect binding assay to MAb-coated target cells (C57BL/6 Con A splenoblasts)/(nu/nu BALB/c splenocytes). c: Molecular weight estimated by immunoprecipitation of lactoperoxidase ^{125}I-labelled C57BL/6 Con A splenoblasts followed by reduction and SDS-PAGE. d: Inhibition of specific ^{51}Cr release from BN rat lymphoma target cells. e: Found to recognize an allospecificity present on C57BL/6 and absent on BALB/c mouse splenocytes. f: Springer[8]. g: Omary et al.[17]; Siadak and Nowinski[20]; Kürzinger et al.[6]. h: Ledbetter and Herzenberg[9].

Group	MAb	Ig Heavy Chain[a]	Binding Ratio T/B[b]	Specificity, MW[c]	No. of Killing Experiments
A	M5/24	γ2b	7	Lyt-2, 35K	5
	M5/35		4	115K	2
	M5/49	γ2a	16	Thy-1, 25K	5
	M5/54	γ2c	11	Thy-1, 25K	4
	M5/56	γ2c	10	Thy-1, 25K	2
	M5/69	γ2a	9		2
	M5/78	γ2b	4		2
	M5/106	μ	4	allo[e], Ly6	2
	M7/5	μ	14		2
	M7/7	γ2a	7	140K, 250K	2
	M7/8	μ	7		2
	M7/14	γ2a	6	LFA-1, 180K, 95K	17
	M7/20	μ	6	60K	3
	M7/21	γ2b	5	allo[e] H-2, 46K, 12K	3
B	M5/114	γ2b	2	Ia, 35K, 28K	2
	M7/81	γ2b	2	Ia, 35K, 28K	3
	M7/83	γ2b	2	Lgp100, 95K	3
C	M7/86	μ	2		3
D	M1/9.3[f]	γ2a	2	CLA, Ly5[g], ~200K	4
	M1/89.18[f]	γ2b		CLA, Ly5[g], ~200K	3
	M1/42[f]	γ2a	2	H-2, 46K, 12K	3
	M1/84[f]	γ2a		most leucocytes, 46K	3
E	53.7[h]	γ2a		Lyt-1	4
	53.6[h]	γ2a		Lyt-2	6



defined by these MAb is expressed as polypeptide chains of $M_r = 30{,}000$–$35{,}000$ (Fig. 2, lane 7) on 11 % of spleen cells, as previously described[9,16].

The M7/14 antibody, defining the second antigen, consistently gave an average of 90 % inhibition of cell-mediated lysis in 17 different experiments (Fig. 1). Blockade by M7/14 MAb is produced both by antibody in culture supernatants and by Ig purified to homogeneity, and is extremely potent, occurring at concentrations as low as 600 ng antibody/ml. The M7/14 MAb recognizes an antigen containing polypeptide chains of $M_r = 180{,}000$ and 95,000 (Fig. 2). This distinguishes it from other

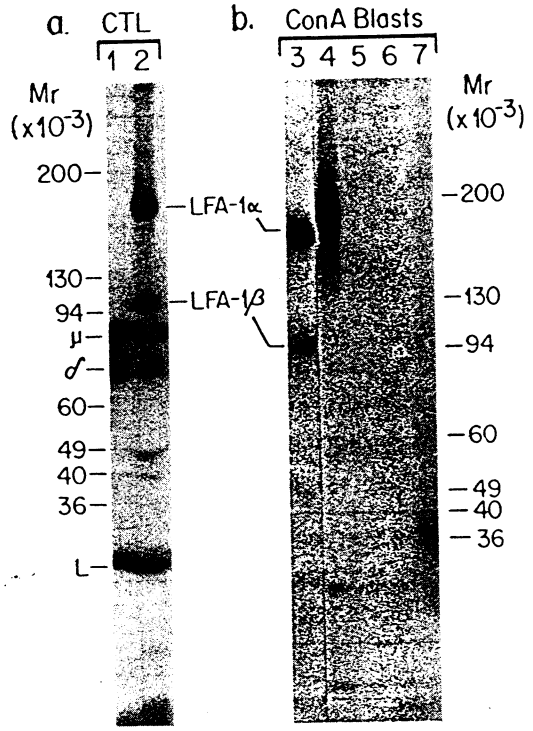


Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of LFA-1, Ly-5, and Lyt-2,3 immunoprecipitated by MAb. Spleen cells from B6 mice primed with BN lymphoma cells and restimulated in vitro (CTL, a), or B6 spleen cells stimulated with 2 μg/ml of concanavalin 1 for 3 days (Con A blasts, b) were surface labeled with ^{125}I using Iodogen (Pierce Chem.). Cell lysates were immunoprecipitated with NSI supernatant plus added normal rat IgG as control (lanes 1 and 6), 20 μg of M7/14 IgG (lanes 2 and 3), or culture supernatants containing the M1/9.3 anti-Ly-5 (or common leukocyte antigen) MAb (lane 4), the non-precipitating M7/87 MAb (lane 5), or the anti-Lyt-2,3 M5/24 MAb (lane 7), and addition of rabbit anti-rat IgG. Reduced samples were subjected to SDS-PAGE in a 5-15 % polyacrylamide gradient gel, and autoradiography. In addition to specifically precipitated LFA-1 α and β chains, mouse μ, δ, and L chains from B cells in the CTL preparation (a) were precipitated by cross-reaction with rabbit anti-rat IgG. Mouse sIg was absent in (b) since the lysate was precleared with rabbit anti-rat IgG and anti-mouse IgG.

previously described CTL surface components, including Ly-5[17] (Fig. 2, lane 4), T145[18], and Lyt-2,3[10–16] (Fig. 2, lane 7). This novel antigen has been designated LFA-1 (lymphocyte function-associated antigen one).

The MAb to LFA-1 also inhibits allo- and anti-modified self CTL-mediated killing, the mixed lymphocyte reaction (MLR), and antigen-specific proliferative T-cell responses (Table 1). Blockade of CTL function by M7/14 is due to inhibition of CTL-target conjugate formation (Table 1). The B-cell plaque-forming cell (p.f.c.) response to NP–Ficoll and mitogenic response to LPS are unaffected by M7/14. T-dependent B-cell p.f.c. responses are inhibited (Table 1). In contrast to anti-LFA-1, inhibition of T-cell responses is not given by anti-Thy-1 or anti-H-2

TABLE 1

Site number and effect on lymphocyte functions of monoclonal antibodies to LFA-1, Thy-1, and H-2

Antigen:	LFA-1	Thy-1	H-2
Monoclonal antibody:	M7/14	M5/49 or M5/56[a]	M1/42 or M7/21[a]
	Sites/positive cell[b] ($\times 10^4$)		
Spleen cells	1.5	21	10
Secondary in vitro B6 anti-P815 CTL	7.2	75	18
	Inhibition[c], (%)		
Xenogeneic B6 anti-BN rat CTL-mediated killing[d]	88	20[h]	20
Allogeneic B6 anti-P815 CTL-mediated killing[d]	73	–8	ND
Anti-TNP modified-self CTL-mediated killing[d]	100	46[h]	ND
Mouse CTL-rat tumor target cell conjugate formation[e]	84	ND[h]	ND
Allogeneic MLR, B6 anti-BALB/c[f]	86	0	0
Xenogeneic MLR, B6 anti-(Lewis × BN) rat[f]	95	0	0
Antigen-specific T cell proliferation[f]	96	6	ND
T-dependent anti-SRBC plaque-forming cells[g]	64	ND	0
T-independent anti-NP– Ficoll plaque-forming cells[g]	6	ND	12
B-cell LPS-stimulated blastogenesis[f]	0	1	13

[a] Results for either antibody, or if available, average for both antibodies.

[b] Determined by saturation immunofluorescence flow cytometry and expressed relative to published H-2 site number [21].

[c] MAb culture supernatant was present at a final concentration of 5–20% during CTL assays or in vitro cultures.

[d] Measured by specific ^{51}Cr release.

[e] Measured by microscopic examination.

[f] Measured by [^{3}H]thymidine incorporation.

[g] After in vitro culture.

[h] M5/49 agglutinated CTL, while M7/14 produced little agglutination.

MAb of the same subclass which bind to a higher number of sites on the cell surface (Table 1). This is an important control, because it demonstrates that inhibition is due to binding to a specific cell surface site (LFA-1) rather than to blanketing of the cell surface by a large number of antibody molecules.

LFA-1 is a widely distributed leukocyte antigen which is found on B cells, T cells, and 80 % of nucleated bone marrow cells as shown by immunofluorescent flow cytometry. Normal splenic T cells express 3-fold more LFA-1 than B cells, and expression increases relative to H-2 after Con A stimulation or in vitro CTL generation[6].

Discussion

The M7/14 MAb inhibits a broad range of T cell-functional responses. It inhibits the formation of adhesions between CTL and target cells. Its presence on B cells suggests that LFA-1 is not the receptor with which T cells recognize specific antigens. Our working hypothesis is that LFA-1 participates in a step closely linked to the MHC-restricted interaction, i.e. recognition by CTL of H-2 on target cells and by T-helper cells of Ia on macrophages[19]. The finding that M7/14 MAb binds to B cells but has no effect on T-independent p.f.c. responses and LPS mitogenesis lends further support to the idea that M7/14 MAb which do not involve cellular interactions, blocks crucial interactions between cells.

This study illustrates the usefulness of MAb for probing molecules of functional importance in intact cells. Two antigens, LFA-1 and Lyt-2, have been suggested by blocking experiments to participate in the CTL killing pathway. LFA-1 was identified for the first time here and also appears important for a number of other T cell-functional responses. In contrast, a large number of other CTL surface antigens do not appear to be essential participants in CTL-mediated killing. The use of MAb to define the surface molecules associated with CTL function, as well as their use in more detailed biochemical and functional experiments, provides a promising means of analyzing complex T-cell effector functions at the molecular level.

Acknowledgements

This work was supported by USPHS grants CA-14723, AI-00233, CA-31798, and AI-18003. Dr. Davignon was supported by a fellowship from the Massachusetts Division of the American Cancer Society.

References

1 Martz, E., 1977, Mechanism of specific tumor cell lysis by alloimmune T-lymphocytes: resolution and characterization of discrete steps in the cellular interaction. Contemp. Top. Immunobiol. 7, 301.

2 Berke, G., 1980, Interaction of cytotoxic T lymphocytes and target cells. Prog. Allergy 27, 69.
3 Henney, C.S., 1977, T-cell-mediated cytolysis: an overview of some current issues. Contemp. Top. Immunobiol. 7, 245.
4 Davignon, D., E. Martz, T. Reynolds, K. Kürzinger and T.A. Springer, 1981, Lymphocyte function-associated antigen 1 (LFA-1): a surface antigen distinct from Lyt-2,3 that participates in T lymphocyte-mediated killing. Proc. Natl. Acad. Sci. U.S.A. 78 (in press).
5 Davignon, D., E. Martz, T. Reynolds, K. Kürzinger and T.A. Springer, 1981, Monoclonal antibody to a novel lymphocyte function-associated antigen (LFA-1): mechanism of blocking of T lymphocyte-mediated killing and effects on other T and B lymphocyte functions. J. Immunol. 127 (in press).
6 Kürzinger, K., T. Reynolds, R.N. Germain, D. Davignon, E. Martz and T.A. Springer, 1981, A novel lymphocyte function-associated antigen (LFA-1): cellular distribution, quantitative expression, and structure. J. Immunol. 127 (in press).
7 Springer, T., G. Galfré, D.S. Secher and C. Milstein, 1978, Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. Eur. J. Immunol. 8, 539.
8 Springer, T.A., 1980, Cell-surface differentiation in the mouse. Characterization of "jumping" and "lineage" antigens using xenogeneic rat monoclonal antibodies. In: Monoclonal Antibodies, eds. R.H. Kennett, T.J. McKearn and K.B. Bechtol (Plenum, New York) p. 185.
9 Ledbetter, J.A. and L.A. Herzenberg, 1979, Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47, 63.
10 Shinohara, N. and D.H. Sachs, 1979, Mouse alloantibodies capable of blocking cytotoxic T-cell function. I. Relationship between the antigen reactive with blocking antibodies and the Lyt-2 locus. J. Exp. Med. 150, 432.
11 Nakayama, E., H. Shiku, E. Stockert, H.F. Oettgen and L.J. Old, 1979, Cytotoxic T cells: Lyt phenotype and blocking of killing activity by Lyt antisera. Proc. Natl. Acad. Sci. U.S.A. 76, 1977.
12 Fan, J., A. Ahmed and B. Bonavida, 1980, Studies on the induction and expression of T cell-mediated immunity. X. Inhibition by Lyt-2,3 antisera of cytotoxic T lymphocyte-mediated antigen-specific and non-specific cytotoxicity: evidence for the blocking of the binding between T lymphocytes and target cells and not the post-binding cytolytic steps. J. Immunol. 125, 244.
13 Hollander, N., E. Pillemer and I. L. Weissman, 1980, Blocking effect of Lyt-2 antibodies on T cell functions. J. Exp. Med. 152, 674.
14 Shinohara, N., U. Hämmerling and D. H. Sachs, 1980, Mouse alloantibodies capable of blocking cytotoxic T cell function. II. Further study on the relationship between the blocking antibodies and the products of the Lyt-2 locus. Eur. J. Immunol. 10, 589.
15 Sarmiento, M., A. L. Glasebrook and F. W. Fitch, 1980, IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt 2 antigen block T cell-mediated cytolysis in the absence of complement. J. Immunol. 125, 2665.
16 Durda, P. J. and P. D. Gottlieb, 1976, The Ly-3 antigens on mouse thymocytes: Immune precipitation and molecular weight characterization. J. Exp. Med. 144, 476.
17 Omary, M. B., I. S. Trowbridge and M. P. Scheid, 1980, T200 cell surface glycoprotein of the mouse. Polymorphism defined by the Ly-5 system of alloantigens. J. Exp. Med. 151, 1311.
18 Kimura, A., H. Wigzell, G. Holmquist, B. Ersson and P. Carlsson, 1979, Selective affinity fractionation of murine cytotoxic T lymphocytes (CTL). Unique lectin specific binding of the CTL-associated surface glycoprotein, T 145. J. Exp. Med. 149, 473.
19 Paul, W. E. and B. Benacerraf, 1977, Functional specificity of thymus-dependent lymphocytes. A relationship between the specificity of T lymphocytes and their functions is proposed. Science 195, 1293.

20 Siadak, A. W. and R. C. Nowinski, 1980, Identification of Ly-5 and T200 antigens on identical cell surface proteins. J. Immunol. 125, 1400.

21 Liberti, P. A., C. J. Hackett and B. A. Askonas, 1979, Influenza virus infection of mouse lymphoblasts alters the binding affinity of anti-H-2 antibody: requirement for viral neuraminidase. Eur. J. Immunol. 9, 751.