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BLOCKING OF CTL-MEDIATED KILLING BY MONOCLONAL ANTIBODIES TO LFA-1 AND LYT-2,3

I. Increased Susceptibility to Blocking after Papain Treatment of Target Cells[1]

STANISLAW H. GROMKOWSKI,[2] WYRTA HEAGY, FRANCISCO SANCHEZ-MADRID,[3] TIMOTHY A. SPRINGER,[4] AND ERIC MARTZ[5]

From the Department of Microbiology, University of Massachusetts, Amherst, MA 01003; and the Laboratory of Membrane Immunochemistry, Sidney Farber Cancer Institute, Harvard Medical School, Boston, MA 02115

It is now established that monoclonal antibodies (MAb) against LFA-1 and Lyt-2,3 antigens on cytolytic T lymphocytes (CTL) block killing function in the absence of C. It has been suggested that the blocking is inversely related to CTL-target affinity. In this report, we studied the effect of papain pretreatment of target cells, because papain is known to remove H-2 and to render target cells more resistant to allospecific CTL. CTL-target conjugate formation was weaker with papain-treated target cells (based on reduced post-dispersion lysis in dextran-containing medium). The concentration of MAb required to produce 40 to 60% inhibition of ^{51}Cr release (2-hr assay) was reduced four to 29-fold for αLFA-1 and 64 to 114-fold for αLyt-2,3. Papain, however, did not induce blocking by MAb to other CTL antigens such as Thy-1, H-2, and T200. Flow cytometric analysis confirmed that papain selectively removed more than 95% of H-2. In kinetic studies of removal and recovery, H-2 density and conjugate formation correlated well with each other. Sensitivity to blocking was not as well correlated, raising the possibility that an unidentified papain-sensitive target cell molecule other than H-2 plays an important role in CTL-target interaction.

Cytolytic thymus-derived lymphocytes (CTL)[6] are specialized cells, the recognized function of which is immune killing. Their target cells are virally or chemically modified autologous cells, tumor cells, or grafted allogeneic cells. CTL are thought to play a major role in resistance to viral infections (1); their role in tumor elimination and graft rejection is less clear-cut (2). The molecular basis for CTL-mediated lysis of target cells is unknown; however, assays have been developed that can resolve killing into three events: recognition-adhesion (producing a strongly adhering CTL-target cell conjugate), lethal hit delivery (target cell is programmed to lyse), and killer cell-independent lysis (3). Recognition of specific antigen is required to initiate these events. Typically, class I MHC[6] antigens (H-2 in the mouse) are recognized in allogeneic killing. Enzyme-induced removal of H-2 inhibits specific allogenic CTL-mediated killing (4, 5). Binding of antibody to the H-2 antigen blocks killing and prevents conjugate formation (6), and such antibody detaches preformed conjugates (7).

Blocking of CTL-mediated killing can also be accomplished at the effector cell level. Murine CTL-mediated killing is blocked (in the absence of C) by monoclonal antibodies (MAb) to two molecules present on CTL plasma membranes, Lyt-2, 3 and LFA-1 (8–10). In contrast, MAb directed toward more than a dozen other molecules present on the CTL plasma membrane have minimal or no effect on killing (11). LFA-1 consists of two noncovalently associated polypeptides of 180,000 and 95,000 m.w. and has structural homologies to Mac-1, a macrophage molecule associated with C3bi receptor function (12). MAb to the Lyt-2, 3 and LFA-1 molecules block the recognition-adhesion step in killing, and it has been proposed that these molecules are involved in strengthening CTL-target adhesion (8, 13). The exact role of the Lyt-2, 3 and LFA-1 molecules in killing, however, is not known, nor is the mechanism of blocking by MAb to these molecules (10).

MacDonald and co-workers (14, 15) found that CTL clones differ considerably in their susceptibility to blocking by the same αLyt-2, 3 MAb used in the present study. These differences were not attributable to differences in Lyt-2, 3 density on the CTL. It was suggested that CTL functional affinity might be inversely related to susceptibility to blocking. Consistent with this proposal, we found that primary CTL were more easily inhibited by αLFA-1 than were secondary CTL, and that lectin-dependent nonspecific killing was more easily inhibited at low than at high lectin concentrations (13). These observations led us to surmise that experimental reductions in CTL-target functional affinity might increase susceptibility to blocking.

In this paper, we report that pretreatment of P815 target cells with papain, which drastically reduces the H-2 density on these target cells, increases by more than 10-fold the susceptibility of CTL-mediated killing to blockade by MAb to Lyt-2, 3 or LFA-1. These observations represent, to our knowledge, the first experimental modulation of the blocking of CTL-mediated killing by MAb directed against CTL membrane proteins.

MATERIALS AND METHODS

Media. Hybridoma growth medium was Dulbecco's modification of Eagle's minimal essential medium supplemented with L-glutamine (final 4 mM), 1 mM

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[2] Address correspondence to Mr. Gromkowski, Department of Microbiology, University of Massachusetts, Amherst, MA 01003.
[3] Recipient of a Fogarty International Fellowship.
[4] Recipient of an American Cancer Society Junior Faculty Award.
[5] Recipient of Research Career Development Award AI-00458 from the National Institute of Allergy and Infectious Diseases.
[6] Abbreviations used in this paper: CTL, cytolytic T lymphocyte; LFA-1, lymphocyte function-associated antigen one; MAb, monoclonal antibody; HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; ETM, enzyme treatment medium; PBES, phosphate buffer in Earle's saline; FI, fluorescence intensities; MHC, major histocompatibility complex.

sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, and 5% fetal calf serum (FCS). Assay medium (L15HGS) was Liebowitz-15 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10 mM HEPES,[6] 11 mM glucose, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FCS. Enzyme treatment medium (ETM), pH 7.4, was bicarbonate-free Earle's saline containing 5 mM phosphate buffer (PBES) supplemented with 15 mM HEPES, 10 to 30 mM L-cysteine (Sigma Chemical, St. Louis MO), 2% FCS, and papain.

Mice. Mice were obtained from the Jackson Laboratory (C57BL/6, Bar Harbor, ME) or Cumberland View Farms ($CD2F_1$ = (BALB/c × DBA/2)F_1, Clinton, TN), or were first generation offspring of such mice bred in our department. Mice were used at 2 to 6 mo of age.

Target cells and ^{51}Cr labeling. DBA/2 P815 mastocytoma cells were maintained as i.p. ascites in $CD2F_1$ mice. For 1 to 2 days before use as target cells, P815 cells were grown in culture in RPMI 1640 plus 10% FCS (GIBCO) and were harvested from these cultures at $<1 \times 10^6$ cells/ml.

For ^{51}Cr labeling, 100 μCi $Na_2{}^{51}CrO_4$ (in 0.1 to 0.2 ml isotonic saline) were added to 0.5 ml L15HGS containing 1×10^7 P815 cells. The mixture was incubated 1 hr at 37°C, (with vortex resuspension every 15 to 20 min). Cells were rinsed (two centrifugations), resuspended in 12 ml L15HGS, and incubated at 37°C until needed (15 to 45 min) in a 10-cm polystyrene culture dish. Cells were then rinsed, counted, and treated with papain.

Effector cells. C57BL/6J mice were primed by injection of 10^7 P815 cells i.p. One to 6 mo later, splenocytes from primed mice were used as responder cells in cultures stimulated with irradiated $CD2F_1$ splenocytes. Secondary CTL were harvested at 4 to 6 days (for details, see References 11 and 16).

Monoclonal antibodies-(MAb). M17/4.4.4 αLFA-1, M12/7.2 αLyt-2, 3, and M15/5.2.4 αLyt-2, 3 MAb (17), 53.6 αLyt-2, 3 MAb (18), M1/42.3.9.8 αH-2, and M1/9.3.4 αT200 Mab (19) were described and characterized previously. M12/7 and M15/5 are IgG2b; the remainder are IgG2a. MI/69.16.11HK (20), used as a control MAb, is an IgG2b from a hybridoma clone that no longer synthesizes the original rat-specific light chain; only the rat heavy chain and mouse nonspecific κ-chain from the NSI cell remain. This MAb does not bind to murine cells. Hybridoma cells were grown in our laboratory. Culture supernatants were harvested and contained 30 to 60 μg/ml IgG estimated by Mancini radial immunoprecipitation (9). Materials used in these experiments were ammonium sulfate precipitates dialyzed against 0.1 M Tris, pH 7.8, which resulted in 25 to 50-fold concentrations (thus Ig was 1 to 3 mg/ml). Precipitates were stored at −20°C, and in some cases were purified by a single pass over a DEAE-Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA) column in 0.02 M K_2HPO_4, pH 8.0, with 0.02% sodium azide. SDS-PAGE analysis showed that the first protein peak was essentially pure Ig, it was dialyzed against isotonic saline containing 10 mM HEPES, pH 7.2, before use.

Papain treatment of P815 cells. Papain (Sigma; twice crystallized) was dialyzed against PBES and stored at −20°C at 14 mg/ml (based on supplier's label). Then, 0.5 to 1.0×10^7 ^{51}Cr-labeled P815 cells were incubated at 37°C in 1 ml ETM in 17 x 100 mm polystyrene test tubes, with gentle vortexing every 10 to 15 min. Control cells were treated identically in ETM minus papain (except in the experiment shown in Figure 1, in which control cells were not pretreated). Cells were then rinsed by centrifugation three times in L15HGS, and clumps were removed by pouring the suspension through 30-μm nylon mesh (Small Parts Inc., Miami, FL). Cells were then counted for use as targets (viability by trypan blue exclusion exceeded 95%).

^{51}Cr-release assay and inhibition by MAb. Assay medium was L15HGS. One to 3×10^4 effector cells in 50 μl were mixed with 50 μl MAb in a 12 x 75 mm polystyrene test tube, and MAb was allowed to react with the cells for 30 min at 37°C. Tubes were then held at 0°C, while 1 to 3×10^4 ^{51}Cr-labeled (and where specified, papain-pretreated) P815 cells were added in 50 μl. Tubes were vortexed and centrifuged 3 min at 500 × G, 2 to 10°C, then incubated 2 hr 37°C. Ice-cold isotonic saline (2 ml) was then added to each tube, tubes were centrifuged at 2 to 10°C for 5 min, 1300 × G, and supernatants containing released ^{51}Cr were decanted and counted with a gamma spectrometer (Beckman Instruments, Fullerton CA). Percentage corrected release was calculated as $100(e - c)/(1 - c)$, in which e represents the experimental release and c is the spontaneous release in control tubes in which effector cells were omitted. Spontaneous release was 6 to 20%. Percentage inhibition of ^{51}Cr release was calculated as $100(n - m)/n$ in which m is the corrected release in MAb-containing tubes, and n is that in no-MAb controls. Assays were done in duplicate.

Flow cytofluorometry. Relative antigen density on P815 cells was determined as follows. First, 1×10^6 cells were incubated with 50 to 150 μg MAb in 0.1 ml L15HGS (5% FCS) for 30 min at 0°C. After two centrifugations to remove unbound primary antibody, cells were resuspended in 0.1 ml containing 100 to 200 μg/ml of fluorescein-conjugated rabbit $F(ab')_2$ anti-rat IgG (FITC/protein = 2.3 mg/g, Cappel Laboratories, Cochranville, PA). Anti-mouse antibodies had been removed from this reagent by passing over a mouse Ig-Sepharose column (20). Cells were incubated for 30 min at 0°C. Unbound secondary antibody was then removed by two centrifugations, and cells were stored for up to several weeks in 1% paraformaldehyde in PBS at 2 to 8°C.

Fluorescence distributions were obtained for 50,000 cells with an FACS-II (using a logarithmic amplifier), and were recorded and transported with a portable flexible magnetic disk unit (Datamate II, Western Telematic Industries, Santa Ana, CA). Plots and experimental mean channel numbers for P815 (MC_e) were obtained with a CP/M (Digital Research, Pacific Grove, CA) microcomputer. Mean channel numbers were converted to fluorescence intensities (FI) relative to glutaraldehyde-fixed sheep red blood cells (SRBC) by using a graph of log of mean channel for glutaraldehyde-fixed SRBC *vs* FACS gain (in verified two-fold steps) for each photomultiplier voltage used. The relative gain (G_r) at which glutaraldehyde-fixed SRBC gave the MC_e was read from the graph. FI was then calculated as G_r/G_e, in which G_e is the gain used in obtaining the MC_e. Corrected FI was then calculated by subtracting the FI of a control sample from the FI of the experimental. Typical controls are given in Table II.

Post-dispersion lysis. Briefly (for details, see References 3 and 21), 50 μl containing 3×10^4 P815 cells plus 50 μl containing 1.5×10^5 lymphocytes were mixed at 0°C in 13 × 100 mm polystyrene test tubes. Tubes were centrifuged 3 min at 500 × G, 2 to 10°C, then incubated for 5 min at 37°C to allow conjugate formation. Then, 2 ml L15HGS containing 10% dextran T500 (Pharmacia, Piscataway NJ) were added, the cells were dispersed by vortexing, and tubes were incubated 3 hr at 37°C. To harvest released ^{51}Cr, 2 ml isotonic saline were added and thoroughly mixed with the dextran by vortexing. Tubes were then centrifuged and supernatants were decanted and counted as above. Two controls were always included. Spontaneous release was determined in tubes from which effector cells were omitted, and was 9 to 20%. The efficacy of dispersion was verified, and the "no adhesion" level of post-dispersion lysis was determined in tubes that did not receive the 5-min 37°C predispersion incubation.

RESULTS

Blocking properties of αLFA-1 (M17/4) and αLyt-2, 3 (53.6) MAb. M17/4 is a recently obtained rat α-mouse LFA-1 MAb (IgG2aκ) that was identified by screening hybridomas for the ability of their MAb-containing supernatants to inhibit CTL-mediated killing (17). Establishment of αLFA-1 specificity was based on immunoprecipitation of two polypeptide chains (180,000 and 95,000 daltons) indistinguishable in SDS-PAGE from the α- and β-chains precipitated by the MAb that first defined LFA-1 (11), M7/14 (similar designations are coincidental). Additionally, the tissue distribution and cell density of the M17/4 antigen is indistinguishable from that of LFA-1 (17), and M7/14 and M17/4 MAb mutually compete for binding to cells (unpublished results).

M17/4 is a potent blocker of CTL-mediated killing; 200 μg/ml typically inhibits ^{51}Cr release (*in vitro* secondary C57BL/6 αP815) more than 70% (mean 74%, range 56 to 86%, four experiments). Binding of M17/4 to the CTL is sufficient for blocking; pretreating (and washing) target cells with MAb has no effect on killing, whereas pretreating CTL produces significant inhibition (data not shown; similar conclusions were reached for M7/14; Reference 11).

In this allogeneic CTL system, the well-characterized 53.6 αLyt-2,3 MAb of Ledbetter and Herzenberg (18) blocks less well. At 200 μg/ml, 36% inhibition of ^{51}Cr release was observed (range 28 to 50%, seven experiments).

Papain treatment of P815 cells increases susceptibility of CTL-mediated killing to blocking by M17/4 and 53.6. Papain pretreatment of P815 target cells augmented blocking of CTL-mediated killing by MAb as shown in Figure 1. These effects were seen consistently in over 20 experiments. These levels of augmented blocking required papain (e.g., Figs. 1 and 2) together with cysteine (data not shown). The effect of papain can be expressed quantitatively as the decrease in concentration of MAb required to obtain a specified level of inhibition of ^{51}Cr release. For M17/4 αLFA-1, 60% inhibition was achieved with a 10-fold drop in MAb concentration after 30 min pretreatment, and with a 15-fold drop after 60 min (Fig. 2). For 53.6 αLyt-2, 3, 36% inhibition (plateau value for untreated cells) was achieved with a 70-fold drop in MAb concentration after 30 or 60 min pretreatment (Fig. 2). Data quite similar to those in Figure 2 were obtained in 19 additional experiments for αLFA-1 and in 6 additional experiments for αLyt-2, 3 (53.6 Mab). The mean reduction in αLFA-1 MAb concentration was 14-fold (range 4 to 29). The reduction

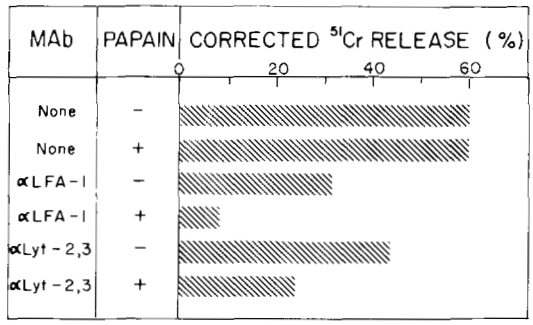

Figure 1. Blocking of killing by αLFA-1 and αLyt-2/3 MAb is increased after papain treatment of the target cells. P815 cells were pretreated in 6 mg/ml papain for 30 min. Effector cells were preincubated with 25 μg/ml M17/4 (αLFA-1) or 200 μg/ml 53.6 (αLyt-2/3) MAb.

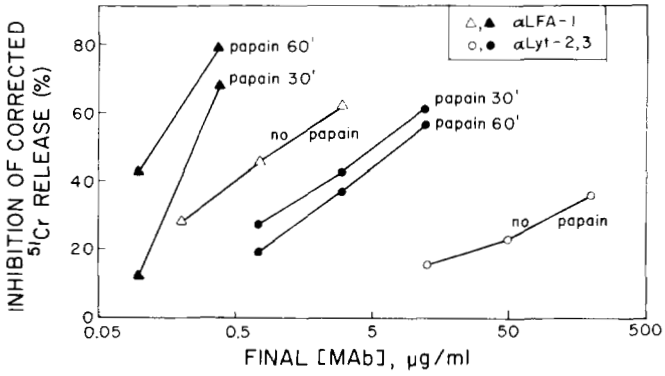

Figure 2. Quantitation of increased blocking with papain-treated target cells. The papain concentration was 6 mg/ml; pretreatment of the P815 target cells was for 30 or 60 min as indicated.

was more difficult to quantitate for αLyt-2, 3 due to the low plateau level of blocking with untreated targets (see Fig. 2), but exceeded 70-fold in three experiments. These comparisons were based on 40 to 60% inhibition levels for αLFA-1 and 28 to 40% for αLyt-2, 3. Similar results were obtained with purified αLFA-1 or αLyt-2, 3 monoclonal Ig (data not shown).

Blocking by two other recently described αLyt-2, 3 MAb, M12/7.2 and M15/5.2.4 (17), was similarly augmented by papain pretreatment of P815 target cells (three experiments, data not shown). The three αLyt-2, 3 MAb gave low plateaus of 43 to 55% inhibition with control target cells, and 70 to 95% with papain-treated targets. These MAb recognize at least two distinct determinants on the Lyt-2, 3 complex (Reference 17, unpublished data).

Papain pretreatment of P815 cells does not induce blocking by "nonblocking" MAb. In contrast to the blocking of CTL-mediated killing that was observed by many workers with αLFA-1 and αLyt-2, 3 MAb (reviewed in Reference 8), we showed previously that MAb to 12 or more other distinct molecules expressed on CTL produce minimal or no inhibition of killing (11). We checked the possibility that papain pretreatment might induce blocking by MAb in this latter "nonblocking" category. MAb to the relatively high-density (22) antigens Thy-1, H-2, and T200 remained unable to block even after papain pretreatment of the P815 cells (Table I). (Although the αH-2 MAb M1/42 binds to the target cell antigen, it is known to be unable to block allogeneic CTL-mediated killing; S. Balk and M. Mescher, unpublished results.)

H-2 is selectively removed by papain treatment. The most straightforward explanation of the papain effect is that it results from reduced H-2 density on the surface of the target cells. Flow cytofluorometry showed that after 30 min in 3 mg/ml papain, P815 cells had lost 96% of their H-2 but only 36% of their T200 (Table II). (This was not a plateau level for T200, because after a 60-min treatment, P815 cells lost 80% of their T200.) In some experiments, 60 min was required for near-complete removal of H-2 antigens (cf. Table III). LFA-1, present in lower density on P815 cells (corrected fluorescence intensity 0.5) was intermediate between H-2 and T200 in sensitivity to papain (data not shown).

Papain treatment of target cells reduces CTL-target adhesion and short-term killing. Removal of more than 90% of the target cell H-2 antigen recognized by the CTL had little effect on killing in a 2-hr ^{51}Cr-release assay, as seen in Figure 1. The largest inhibition in our hands was 28% (data not shown). For reasons unknown to us, this is in contrast to the complete abolition of killing observed by others after papain treatment (4, 5, 23). We reasoned that in our hands recovery from the effects of papain

TABLE I

Papain pretreatment of target cells does not induce blocking of killing at CTL sites other than LFA-1 and Lyt-2, 3[a]

Antigen	(MAb)	Percent Corrected ^{51}Cr Release ([MAb], μg/ml)	
		−papain	+papain
None	(M1/69HK)	63 (200)	70 (200)
T200	(M1/9.3)	64 (200)	68 (200)
Thy-1	(M5/49)	62 (200)	61 (200)
H-2	(M1/42)	64 (200)	66 (200)
LFA-1	(M17/4)	31 (12.5)	25 (0.4)
Lyt-2,3	(53.6)	45 (200)	32 (12.5)

[a] Papain pretreatment of P815 target cells was for 30 min at 6 mg/ml and 37°C.

TABLE II

Papain selectively reduces H-2 density on P815 cells[a]

Antigen	MAb	Papain (mg/ml)	Corrected Fluorescence Intensity[b]	Reduction in Antigen Density (%)
T200	M1/9.3	0	2.5	
H-2	M1/42		2.5	
T200	M1/9.3	1.5	2.0	20
H-2	M1/42		0.5	80
T200	M1/9.3	3.0	1.6	36
H-2	M1/42		0.1	96

[a] P815 cells were treated for 30 min at 37°C in ETM with the indicated concentration of papain.

[b] Corrected fluorescence intensity in glutaraldehyde-fixed SRBC units. P815 cells are Lyt-2, 3-negative, hence 53.6 αLyt-2, 3 MAb was used to establish control levels of fluorescence intensity for correcting experimental values (see *Materials and Methods*). The background fluorescence intensity of the 53.6 MAb-treated P815 cells was 0.8 with or without papain pretreatment. The fluorescence intensity for two other controls, no MAb or M1/69HK nonspecific MAb (see *Materials and Methods*) ranged between 0.7 and 0.9. These controls therefore had a corrected fluorescence intensity of −0.1 to 0.1.

TABLE III

Papain treatment of P815 target cells weakens their specific adhesion formation with CTL[a]

Experiment	Percent Post-dispersion Lysis[b]		Minutes in Papain	Percent Inhibition
	−papain	+papain		
1	55	25	30	55
2	41	22	30	46
	41	7	60	83
3	57	12	30	79
4	38	19	30	50
5	77	27	30	65
6	61	1	30	98

[a] Papain treatments were at 6 mg/ml in Expts. 1 and 2, and 3 mg/ml in Expts. 3–6.

[b] Percent corrected ^{51}Cr release in post-dispersion lysis assay.

might occur within a fraction of the 2-hr assay time, so ^{51}Cr-release assays were conducted for shorter times at proportionately higher effector to target cell ratios. Inhibition was dramatic in a 10-min assay, was reduced in a 30-min assay, and was hardly detectable in a 90-min assay (Fig. 3). This showed that initially papain-treated targets were resistant to killing, but that they recovered their sensitivity to killing within about 1 hr.

Because the specific H-2 antigen removed by papain is necessary for the initial recognition-adhesion step in CTL-mediated killing (6), we expected to see reduced adhesion in the post-dispersion lysis assay (3). In this assay, CTL-target mixtures are dispersed in a relatively large volume of dextran-containing medium. ^{51}Cr release is produced only by those CTL-target conjugates that had adhered strongly enough to resist dispersion, and therefore remain attached in the dextran-containing medium (24). Unattached cells do not establish new contacts in the dextran-containing medium (3). The percentage of target cells that formed dispersion-resistant adhesions in a 5-min incubation was reduced by 50 to 98% for papain-treated target cells (Table III), thus showing that papain treatment reduced the strength of CTL-target adhesion, at least during the first 5 min at 37°C.

P815 target cells were pretreated in various concentrations of papain for 30 min, and the effects on H-2 density, post-dispersion lysis, and susceptibility of killing to blocking with αLFA-1 were monitored. As seen in Figure 4, CTL-target cell adhesion (post-dispersion lysis) decreases in parallel with H-2 density. The concentration of MAb required for 60% inhibition of killing, in contrast, is not parallel with the other two parameters. It is unaffected by the initial 56% reduction in H-2, but then shows a three-fold decrease due to an increase in papain that has minimal further effect on H-2 density. (Such a lack of parallelism was seen in two of three experiments in which post-dispersion lysis and blocking were quantitated, including the experiment shown in Fig. 4.)

Recovery of papain-treated target cells. After papain treatment of P815 cells, the re-expression of H-2 at 37°C was monitored by fluorescence. The H-2 density returned from 10 to 58% of the level on untreated control cells during a 2-hr incubation (Fig. 5). During the same period, the concentration of αLFA-1 MAb required for 55% inhibition of killing increased in a somewhat slower manner from 13 to 35% of the concentration in controls. The ability of the target cells to form dispersion-resistant adhesions in a 5-min incubation started at 50% of the control level in this experiment (cf. Table III) and recovered fully within 1 hr (Fig. 5).

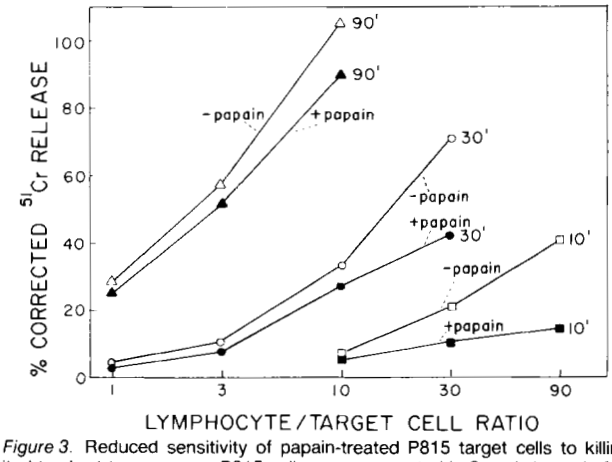

Figure 3. Reduced sensitivity of papain-treated P815 target cells to killing is limited to short-term assays. P815 cells were pretreated in 3 mg/ml papain for 30 min. Total incubation of effectors with targets before harvesting released ^{51}Cr was 10, 30, or 90 min as indicated. Open symbols are for control P815 cells (papain omitted); filled symbols are for papain pretreated P815 cells.

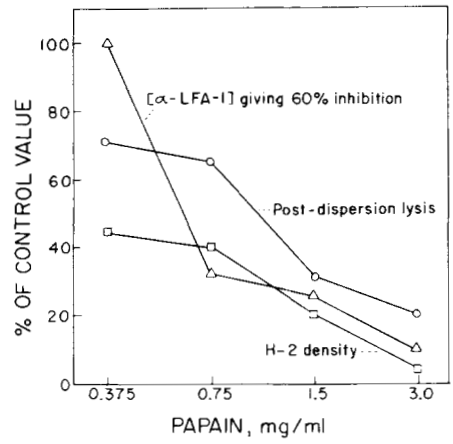

Figure 4. Effect of various papain concentrations during pretreatment of target cells on H-2 density (squares), [αLFA-1 MAb] required for 60% inhibition of killing (triangles), and post-dispersion lysis (circles). P815 cells were pretreated for 30 min with the papain concentrations indicated. Post-dispersion lysis gave 57% corrected release in controls (papain omitted).

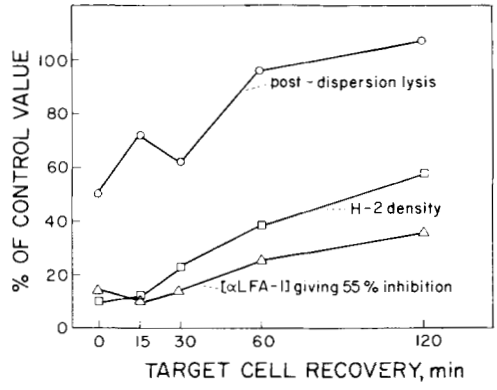

Figure 5. Recovery of P815 cells from papain treatment. P815 cells were treated for 30 min at 37°C with 3 mg/ml papain. Cells were then rinsed and allowed to recover at 37°C in L15HGS for the times indicated. Recovery was calculated as a percentage of the control (papain omitted during pretreatment) levels for MAb required to give 55% inhibition of corrected ^{51}Cr release (triangles), H-2 density (squares), and post-dispersion lysis (circles). MAb was M17/4; 3 μg/ml gave 55% inhibition of killing of control P815 cells (papain omitted). In the obsence of papain, post-dispersion lysis was 38%.

DISCUSSION

The present experiments demonstrate that papain pretreatment of P815 target cells markedly increases the susceptibility of allogeneic CTL-mediated killing to blocking by MAb; the concentration of MAb required to produce 40 to 60% inhibition of ^{51}Cr release (2-hr assay) was reduced up to 29- to 114-fold for αLFA-1 and αLyt-2, 3, respectively.

We also examined the effect of papain on the recognition-adhesion step because it is recognition of the papain-sensitive class I MHC antigens (H-2 in the mouse) that initiates allogeneic killing. Dispersion-resistant CTL-target adhesion formation during the first 5 min of interaction was reduced 50 to 98% when papain-treated P815 cells were used as targets.

The mechanism of the increase in susceptibility of blocking is not known. It has been suggested that Lyt-2, 3 is involved in specific antigen receptor function on T lymphocytes. The idea that Lyt-2, 3 may play some supportive role in recognition of class I MHC antigens (25, 26) was strengthened by recent findings concerning the human homolog of Lyt-2, 3, OKT8/Leu-2a (reviewed in Reference 10). OKT8/Leu-2a appears to be

expressed only on CTL that recognize, or whose recognition is restricted by, class I MHC antigens. MAb to OKT8/Leu-2a block these CTL. CTL that recognize class II MHC antigens express OKT4/Leu-3, which is a blocking site for these CTL, but not anti-class I CTL (and *vice versa*). If Lyt-2, 3 plays a supporting role in the function of the specific antigen receptor, it would be easy to understand the effect of papain on blocking by MAb on the basis of steric interference by the MAb with receptor-H-2 interaction. Such a role seems less likely for LFA-1 in view of its wide tissue distribution (10, 22).

The most straightforward explanation of the papain effect is that it results from reduced H-2 density on the surface of the target cell. We showed by flow cytofluorometry that papain removed 95 to 98% of the surface H-2 on P815 cells; however, the possibility cannot be excluded that the removal of some other molecule(s) is involved in these functional effects.

Indeed, kinetic data suggest differences in H-2 density alone may not account for the increase in susceptibility to blocking. Susceptibility to blocking was less affected by low doses of papain than were adhesion or H-2 density. There was little change in the latter two between 0.375 and 0.75 mg/ml papain, whereas susceptibility to blocking decreased three-fold in the same interval (Fig. 4). (Previous evidence (23) suggests there is no minimum "threshold" level of H-2 necessary for recognition of the target cell.) During recovery, the susceptibility to blocking returned more slowly than did the adhesion or H-2 density (Fig. 5).

This poor correlation between susceptibility to blocking on the one hand and adhesion formation or H-2 density on the other hand is consistent with the possibility that a protease-sensitive non-H-2 target cell molecule plays an important role in killing. This possibility is supported by recent data showing that trypsin pretreatment of target cells produces augmented blocking of allogeneic CTL-mediated killing without affecting H-2 density on the target cells (S. Gromkowski, W. Heagy, and E. Martz, manuscript in preparation).

We suspect that the formation of a CTL-target conjugate involves two steps: a specific immunologic recognition (interaction between the CTL receptor and target H-2) and an adhesion-strengthening process (24), triggered by this recognition.

An attractive possibility is that LFA-1 (and/or Lyt-2,3) is directly involved in the adhesion-strengthening process, perhaps by binding to some (non-H-2) protein ligand on the target cell (8, 13). Enzymatic removal of the target ligand molecule would then impair adhesion strengthening, thereby making blocking easier.

As mentioned above, the premise for this study was the notion that the functional affinity of CTL for the target cells might be inversely related to susceptibility to blocking. In addition to papain pretreatment of target cells, we studied CTL-mediated killing with normal splenocytes as target cells. Normal splenocytes have been demonstrated to have a lower binding affinity for CTL than do P815 cells by using a reversible adhesion assay recently developed by Balk and Mescher (27). In some but not all experiments, killing of spleen cells was more inhibited than was killing of P815 cells (both assays at 28°C to keep spontaneous release low, Reference 28). The increase in blocking with the spleen cell targets was less than with papain-treated P815. Nevertheless, these results are consistent with the notion that CTL-target functional affinity is inversely related to susceptibility to blocking.

The present results to our knowledge, provide the first demonstration of experimental modulation of the blocking of CTL-mediated killing by MAb to LFA-1 and Lyt-2, 3. This provides a new tool to help elucidate the roles of these molecules in CTL function. We are currently investigating the possibility that an external target cell membrane protein other than specific antigen plays a crucial role in CTL-target cell interaction.

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REFERENCES

1. Lin, Y., and B. A. Askonas. 1981. Biological properties of an influenza A virus-specific killer T cell clone inhibition of virus replication *in vivo* and induction of delayed-type hypersensitivity reactions. J. Exp. Med. 154:225.
2. Loveland, B. E., and I. F. C. McKenzie. 1982. Which T cells cause graft rejection? Transplantation 33:217.
3. 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.
4. Thomas, K., H. D. Engers, J.-C. Cerottini, and K. T. Brunner. 1976. Enzymatic removal of H-2 alloantigens from the surface of P815-(X2) mouse tumor cells. Eur. J. Immunol. 6:257.
5. Todd, R. F. 1975. Lymphocyte-mediated cytolysis of allogeneic tumor cells *in vitro* III. Enzyme sensitivity of target cell antigens. Cell. Immunol. 20:257.
6. Todd, R. F., III, R. D. Stulting, and G. Berke. 1973. Mechanism of blocking by hyperimmune serum of lymphocyte-mediated cytolysis of allogeneic tumor cells. Cancer Res. 33:3203.
7. Balk, S. P., and M. F. Mescher. 1981. Specific reversal of cytolytic T lymphocyte-target cell interaction. J. Supramol. Struct. Cell. Biochem. 16:43.
8. Martz, E., D. Davignon, K. Kürzinger, and T. A. Springer. 1982. The molecular basis for cytolytic T lymphocyte function: analysis with blocking monoclonal antibodies. Adv. Exp. Med. Biol. 146:447.
9. Springer, T. A. 1982. Quantitation of hybridoma immunoglobulins and selection of light chain loss variants. Methods Enzymol. In press.
10. **Martz, E., W. Heagy, and S. H. Gromkowski. 1983. The mechanism of CTL-mediated killing: monoclonal antibody analysis of the roles of killer and target cell membrane proteins. Immunol. Rev. 72:415.**
11. **Davignon, D., E. Martz, T. Reynolds, K. Kürzinger, and T. A. Springer. 1981. Lymphocyte function-associated antigen one (LFA-1): a surface antigen distinct from Lyt-2/3 that participates in T lymphocyte-mediated killing. Proc. Natl. Acad. Sci. USA 78:4535.**
12. Beller, D. I., T. A. Springer, and R. D. Schreiber. 1982. Anti-Mac-1 selectively inhibits rosetting mediated by the type III complement receptor (CR3). J. Exp. Med. 156:1000.
13. Springer, T. A., D. Davignon, M.-K. Ho, K. Kurzinger, E. Martz, and F. Sanchez-Madrid. LFA-1 and Lyt-2, 3, molecules associated with T lymphocyte-mediated killing; and Mac-1, and LFA-1 homologue associated with complement receptor function. Immunol. Rev. 68:111.
14. MacDonald, H. R., N. Thiernesse, and J.-C. Cerottini. 1981. Inhibition of T cell-mediated cytolysis by monoclonal antibodies directed against Lyt-2: heterogeneity of inhibition at the clonal level. J. Immunol. 126:1671.
15. MacDonald, H. R., A. L. Glasebrook, and J.-C. Cerottini. 1982. Clonal heterogeneity in the functional requirement for Lyt-2/3 molecules on cytolytic T lymphocytes: analysis by antibody blocking and selective trypsinization. J. Exp. Med. 156:1711.
16. 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 blockade of T lymphocyte-mediated killing and effects on other T and B lymphocyte functions. J. Immunol. 127:590.
17. Sanchez-Madrid, F., D. Davignon, E. Martz, and T. A. Springer. 1982. Functional screening for antigens associated with mouse T lymphocyte-mediated killing yields antibodies to Lyt-2, 3 and LFA-1. Cell. Immunol. 73:1.
18. Ledbetter, J. A., and L. A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47:63.
19. Springer, T. A. 1980. Cell-surface differentiation in the mouse. Characterization of "jumping" and "lineage" antigens using xenogeneic rat monoclonal antibodies. *In* Monoclonal Antibodies. R. H. Kennett, T. J. McKearn, and K. B. Bechtol, eds. Plenum, New York. P. 185.
20. Springer, T. A. 1980. Quantitation of light chain synthesis in myeloma × spleen cell hybrids and identification of myeloma chain loss variants using radioimmunoassay. J. Immunol. Methods 37:139.
21. Martz, E. 1975. Early steps in specific tumor cell lysis by sensitized mouse T-lymphocytes I. Resolution and characterization. J. Immunol. 115:261.
22. 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. 127:596.
23. Kuppers, R. C., Z. K. Ballas, W. R. Green, and C. S. Henney. 1981. Quantitative appraisal of H-2 products in T cell-mediated lysis by allogeneic and syngeneic effector cells. J. Immunol. 127:500.
24. Martz, E. 1980. Immune lymphocyte to tumor cell adhesion: magnesium-sufficient, calcium-insufficient. J. Cell Biol. 84:584.
25. Ledbetter, J. A., W. E. Seaman, T. T. Tsu, and L. A. Herzenberg. 1981. Lyt-2 and Lyt-3 antigens are on two different polypeptide subunits liked by disulfide

bonds. Relationship of subunits to T cell cytolytic activity. J. Exp. Med. 153:1503.
26. MacDonald, H. R., A. L. Glasebrook, O. Acuto, A. Kelso, C. Bron, and J.-C. Cerottini. 1982. Heterogeneity of inhibition of cytolytic T lymphocyte clones by monoclonal anti-Lyt-2/3 antibodies: parallel effects on cytolysis, proliferation, and lymphokine secretion. Adv. Exp. Med. Biol. 146:533.
27. Balk, S. P., and M. F. Mescher. 1981. Specific reversal of cytolytic T cell-target cell functional binding is induced by free target cells. J. Immunol. 127:51.
28. Balk, S. P., and M. F. Mescher. 1981. Cytolytic T lymphocyte mediated chronium-51 release versus spontaneous release from blast and spleen cell targets at low temperature. Cell. Immunol. 65:201.