arthritis reactive with a self-associated antigen. Demonstration in precipitation and immunofluorescence. Arthritis Rheum. 19:711.
14. Nonoyama, M., and J. S. Pagano. 1971. Detection of Epstein-Barr viral genome in nonproductive cells. Nature (New Biol.) 233:103.
15. Nonoyama, M. 1975. Detection of tumor virus genomes by nucleic hybridization. Int. Rev. Exp. Pathol. 14:69.
16. Raab-Traub, N., T. Dambaugh, and E. Kieff. 1980. DNA of Epstein-Barr virus VIII. B95-8, the Prennis prototype, is an unusual deletion derivative. Cell 22:257.
17. Dambaugh, T., D. Beisel, M. Hummel, W. King, S. Ferr, A. Cheung, M. Heller, N. Raab-Traub and E. Kieff. 1980. Epstein-Barr virus (B95-8) DNA. VII. molecular cloning and detailed mapping. Proc. Natl. Acad. Sci. 77:2999.
18. Riccuiti, F., and F. H. Ruddle. 1973. Assignment of nucleoside phosphorylase to D-14 and localization of X-linked loci in man by somatic cell genetics. Nature (New Biol.) 241:180.
19. Yamamoto, K., F. Mizuno, T. Matsuo, A. Tanaka, M. Nonoyama, and T. Osato. 1978. Epstein-Barr virus and human chromosomes: close association of the resident viral genome and the expression of the virus-determined nuclear antigen (EBNA) with the presence of chromosome 14 in human-mouse hybrid cells. Proc. Natl. Acad. Sci. 75:5155.
20. Spira, J., S. Povey, F. Wiener, G. Klein, and M. Anderson-Anvret. 1977. Chromosome banding, isoenzyme studies and determination of Epstein-Barr virus DNA content on human Burkitt lymphoma-mouse hybrids. Int. J. Cancer 20:849.
21. Steplewski, Z., H. Koprowski, M. Anderson-Anvret, and G. Klein. 1978. Epstein-Barr virus in somatic cell hybrids between mouse cells and human nasopharyngeal carcinoma cells. J. Cell Physiol. 97:1.
22. Croce, C. M., M. Shander, J. Martinis, L. Cicinel, G. G. D'Ancona, and H. Koprowski. 1980. Preferential retention of human chromosome 14 in mouse × human B cell hybrids. Eur. J. Immunol. 10:486.
23. Glaser, R., M. Nonoyama, B. Hampar, and C. M. Croce. 1978. Studies on the association of the Epstein-Barr virus genome and human chromosomes. J. Cell Physiol. 96:319.
24. Glaser, R., M. Nonoyama, M. Whitman, and T. Forcino. 1979. Studies on an epithelial/Burkitt hybrid cell line prepared from a lymphoblastoid cell line with one EBV genome equivalent per cell. Virology 98:272.
25. Alspaugh, M. A., and E. M. Tan. 1975. Antibodies to cellular antigens in Sjogren's syndrome. J. Clin. Invest. 55:1067.
26. Baron, D., and J. L. Strominger. 1978. Partial purification and properties of the Epstein-Barr virus-associated nuclear antigen. J. Biol. Chem. 253:2875.

0022-1767/81/1271-0590$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 127, No. 2, August 1981
*Printed in U.S.A.*

# 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[1]

DENISE DAVIGNON,[2] ERIC MARTZ,[3] TIM REYNOLDS, KONRAD KÜRZINGER,[4] AND TIMOTHY A. SPRINGER

*From the Laboratory of Membrane Immunochemistry, Sidney Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, and Department of Microbiology, University of Massachusetts, Amherst, MA 01103*

**The M7/14 monoclonal antibody (MAb) has previously been shown to block xenogeneic cytolytic T lymphocyte-(CTL) mediated killing by binding to the CTL rather than the target cell. It defines a novel surface antigen, termed LFA-1, with polypeptide chains of 180,000 and 95,000 $M_r$ (1). The mechanism of this blockade and the effects of M7/14 on other T and B lymphocyte functional responses have been investigated here. Blockade of CTL-mediated killing is given by purified M7/14 MAb, and is not due to trivial mechanisms such as toxicity or agglutination. M7/14 blocks CTL directed to allogeneic and anti-modified self as well as xenogeneic determinants. Blockade occurs at the recognition-adhesion step, as shown by inhibition of CTL-target cell conjugate formation.**

**M7/14 antibody was also shown to block a variety of other T cell functions: the mixed lymphocyte response (both in xenogeneic and allogeneic systems), antigen-specific T cell proliferation, and T cell-dependent plaque-forming cell responses, but not T cell-independent plaque-forming cell responses or LPS-induced B cell proliferation. Inhibition of both CTL and MLR function was extremely potent, occurring at MAb concentrations as low as 0.5 μg/ml. Inhibition of the MLR occurs in the 1st day of 6-day cultures. The data suggest that LFA-1 participates in or is closely associated with a number of different T lymphocyte functional pathways. LFA-1 appears crucial for the adhesive interaction between CTL and target cells, and it is possible that its importance in T helper and proliferative responses is also related to intercellular interactions.**

In a previous study, we used a panel of 24 monoclonal antibodies (MAb)[5] to probe the relation of cytolytic T lymphocyte (CTL) surface molecules to CTL function (1). These xenogeneic rat anti-mouse MAb defined at least 12 different mouse T lymphocyte cell surface structures, some for the first time. The MAb were tested for their ability to block CTL-mediated killing in the absence of complement. A xenogeneic mouse CTL anti-rat lymphoma tumor cell system was utilized for testing. This ensured that the MAb could not react with the target cells (both of rat origin) and that any observed effects were due to reaction with effector cells. Twenty-one MAb defining at least 10 antigens expressed on T lymphocytes, including Thy-1, H-2, Lyt-1, Ly-4, Ly-6, Lgp100, previously undescribed antigens of 140,000 and 250,000, 115,000, 60,000 and 44,000 relative molecular mass ($M_r$), and other antigens not structurally

Received for publication February 25, 1981.
Accepted for publication April 30, 1981.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported by United States Public Health Service Research Grants CA-14723, CA-31798, and AI-18003, and by a grant from the Upjohn Company.
[2] Postdoctoral fellow of the Massachusetts Division of the American Cancer Society.
[3] Recipient of Research Career Development Award AI-00233 from the National Institute of Allergy and Infectious Diseases. Present address: Department of Microbiology, University of Massachusetts, Amherst, MA 01103.
[4] Postdoctoral fellow of the Deutsche Forschungsgemeinschaft.

[5] Abbreviations used in this paper: B6, C57BL/6J; BN, brown Norway rat strain; BNLφ, BN rat lymphoma; CLA, common leukocyte antigen; CTL, cytolytic T lymphocyte(s); GAT, L-glutamic acid$^{60}$-L-alanine $^{30}$-L-tyrosine$^{10}$; H chain, specific heavy chain; HEPES, *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer; K chain, myeloma κ-chain; L chain, specific light chain; LFA, lymphocyte function-associated antigen; MAb, monoclonal antibody; MAbCS, monoclonal antibody-containing culture supernatant; $M_r$, relative molecular mass (molecular weight); NP, 4-hydroxy-3-nitrophenyl acetyl.

characterized but with distinct cell distributions, had little or no effect on CTL-mediated killing.

Two MAb to the Lyt-2,3 antigen complex of 30,000–35,000 $M_r$ (2, 3) blocked xenogeneic CTL-mediated killing, confirming previous reports with allogeneic CTL (4–9).

Another MAb, M7/14, defined a novel cell surface site for antibody-mediated blockade of CTL function. This MAb gave consistent 90% inhibition of CTL-mediated lysis and immunoprecipitated a previously undescribed lymphocyte surface antigen with polypeptide chains of 180,000 and 95,000 $M_r$. The results suggested the antigen either participated in or was closely associated with the mechanism of cell-mediated killing, and it was designated a lymphocyte function-associated antigen (LFA-1).

In this report, we have studied the mode of inhibition by M7/14 MAb of CTL-mediated killing. We have also characterized the effects of M7/14 MAb on other cellular functions, including allogeneic and anti-modified self CTL-mediated killing, the MLR, antigen-specific T cell proliferation, T-independent and -dependent B cell plaque-forming cell responses, and mitogenesis.

## MATERIALS AND METHODS

*Media and reagents.* For *in vitro* generation of effector cells, the culture medium was RPMI 1640 with the following additions: 2 mM extra L-glutamine, 2 g/liter extra D-glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, $5 \times 10^{-5}$ M 2-mercaptoethanol (Eastman Kodak Co., Rochester, NY), and 2% heat-inactivated fetal calf serum (FCS).

For assay of CTL function, the medium (L15HGS) was L15 medium (Microbiological Associates, Bethesda, MD) containing 5% heat-inactivated fetal calf serum, D-glucose (2 mg/ml), 10 mM HEPES (see Abbreviations) buffer (Grand Island Biological Co., Grand Island, NY), 100 U/ml penicillin, and 100 μg/ml streptomycin. MLR and mitogen cultures were in RPMI 1640 with additions as above except for omission of D-glucose and addition of $5 \times 10^{-5}$ M 2-mercaptoethanol.

*Cells.* Targets were brown Norwegian (BN) rat lymphoma (BNLφ) cells, P815 mastocytoma cells (of DBA/2 origin (10)), and EL-4 lymphoma cells (of C57BL/6 origin (11)). BNLφ were maintained in culture in RPMI 1640 with 5% heat-inactivated FCS. P815 and EL-4 were maintained by serial passage i.p. in $(BALB/c \times DBA/2)F_1$ ($CD2F_1$) (Cumberland View Farms, Clinton, TN) and C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) mice, respectively. Target cells were labeled with $^{51}Cr$ as previously described (12).

C57BL/6 anti-BNLφ (xenogeneic) effector cells were generated by *in vitro* secondary stimulation in culture. Spleens from C57BL/6 mice immunized i.p. with $10^7$ BNLφ viable cells 1 to 3 mo previously were harvested aseptically and minced, and the splenocytes were washed with medium 3 times for use as responders. Stimulators were BNLφ cells, which were irradiated with 1600 R from a Gammacell-40 cesium source Research Irradiator (Atomic Energy of Canada Limited, Ottawa, Canada) and were subsequently washed 3 times. Responders ($8 \times 10^7$) were cultured with $2 \times 10^6$ stimulators in plastic tissue culture flasks (No. 25100, Corning Glass Works, Corning, NY) in a total volume of 25 ml. Flasks were incubated upright at 37°C in a humidified atmosphere of 5% $CO_2$ in air for 4 to 6 days.

Similarly, effector cells against TNP-modified self antigens were generated in 1-way mixed lymphocyte cultures according to a modification of the procedure by Burakoff *et al.* (13).

Cells from *in vitro* cultures were harvested, washed, and resuspended in 2 ml assay medium. Nonviable cells were removed by layering the harvested cells over 3 ml lymphocyte separation medium (Bionetics, Kensington, MD) and centrifuging at 500 × *g* for 7 min at room temperature. Cells were collected from the interface, washed 3 times, and resuspended in medium. Viable cell numbers were determined by trypan blue exclusion.

Mouse alloimmune effector cells were nonadherent peritoneal wash cells from mice that had been immunized i.p. with allogeneic tumor cells as described previously (14).

*Monoclonal antibodies (MAb).* MAb have been previously described (1, 15). All were NSI × rat spleen cell hybrids that had been cloned and subcloned. Subclone designations have been omitted for brevity. NSI supernatant plus 50 μg/ml of normal rat IgG was used as a control.

*CTL assays and inhibition of cell-mediated cytolysis by MAbs.* Effector cells (50 μl) were pretreated with 50 μl monoclonal hybridoma culture supernatant (MAbCS) or medium in 12 × 75 mm plastic tubes (No. 2052, Falcon Plastics) for 30 min at room temperature vortexing at 0 min and 15 min. (The MAbCS had been dialyzed against L15HG medium at 4°C for 24 hr with 2 changes of medium.) On ice, $1 \times 10^4$ of the appropriate $^{51}Cr$-labeled target cells were added in 50 μl. All tubes were vortexed, centrifuged at 4°C for 5 min at 500 × *g*, and then incubated at 37°C for 2 to 4 hr.

*Determination of corrected percentage $^{51}Cr$ release.* After incubation at 37°C, 4 ml of ice-cold 0.9% NaCl were added to each tube. The tubes were vortexed and then centrifuged at 4°C for 10 min at 1600 × *g*. The supernatants were decanted and counted for released $^{51}Cr$ in a gamma spectrometer. Corrected percentage $^{51}Cr$ release was calculated as $100(e - c)/(100 - c)$, where *e* represents the percentage of $^{51}Cr$ released in tubes/wells containing both effector and target cells + supernatant, and *c* the percentage of $^{51}Cr$ released in control tubes/wells in which medium was substituted for the effector cells. Release values are expressed as a percentage of the $^{51}Cr$ released by freezing (in liquid nitrogen) and thawing replicate aliquots of labeled target cells. Isotope counts on punched paper tape were read, and calculations were performed, by a Digital Equipment Corp. 11/70 computer running under UNIX (trade name of the Bell System) at the Harvard School of Public Health.

*MLR and mitogen cultures.* For MLR, C57BL/6J (B6) responder spleen cells ($1.25 \times 10^6$/ml) were cultured with syngeneic cells (control), BALB/c spleen cells, or LBN rat cervical and axillary lymph node stimulator cells ($2.5 \times 10^6$/ml) that had been irradiated with 1500 R. For mitogen responses, B6 spleen cells ($2.5 \times 10^6$/ml) were cultured with no additions (control), 2 μg/ml concanavalin A (Con A; Sigma) or 10 μg/ml *Salmonella typhosis* lipopolysaccharide W (LPS; Difco). MAbCS or IgG, at various dilutions in culture medium (50 μl), were added at culture initiation (200 μl total in 96-well flat-bottom plates). [$^3$H] thymidine (1 μCi) was added after 68 hr (8-hr pulse) for mitogen cultures, or 5 days (18-hr pulse) for the MLR. Other procedures were as in (16).

*Purification and labeling of M7/14 IgG.* M7/14 IgG was purified from growth medium containing 5% FCS by $(NH_4)_2SO_4$ precipitation and DEAE and G-200 chromatography as previously described (17). [$^{14}$C] leucine was incorporated into secreted Ig according to Cotton *et al.* (18) using 5 μCi of [$^{14}$C] leucine (New England Nuclear, Boston, MA) per milliliter of labeling medium.

## RESULTS

*Characteristics of inhibition by M7/14 of CTL-mediated killing.* The M7/14 hybridoma secretes an IgG2a Ig containing specific heavy (H) and light (L) and myeloma κ chains (K) (Fig. 1*A*) into culture medium to a concentration of 94 μg/ml as determined by Mancini radial immunodiffusion against rabbit anti-rat Fab. Previous work with M7/14 utilized MAbCS, but in this report purified M7/14 IgG has also been used. M7/14 IgG was purified by $(NH_4)_2SO_4$ precipitation and DEAE and G-200 chromatography to homogeneity (Fig. 1*B*). The purified Ig was tested for inhibition of

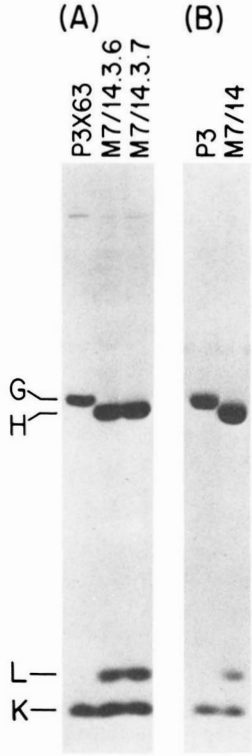


*Figure 1.* SDS-PAGE characterization of [$^{14}$C]-labeled M7/14 supernatants and of purified M7/14 IgG. (*A*) [$^{14}$C]-labeled supernatants (20 μl) of 2 different subclones of M7/14, M7/14.3.6, and M7/14.3.7, were analyzed on 10% SDS-PAGE (32) under reducing conditions. [$^{14}$C]-labeled supernatant from the myeloma line P3-X63-Ag8 which shares the same κ-chain as NSI, was used as a standard. (*B*) Four micrograms of unlabeled purified M7/14 IgG and P3 IgG were analyzed as above. The bands were visualized by staining with Coomassie Blue (33). G, K: γ- and κ-chain of the myeloma line; H,L: specific heavy and light chain of M7/14.

xenogeneic CTL-mediated killing of BNLφ cells, which was previously shown to be mediated by classical Thy-1$^+$, Lyt-2$^+$, antigen-specific CTL (1). The purified Ig inhibited killing by 90%, as previously reported for the MAbCS (1), even when high effector: target ratios or highly active CTL preparations were tested that gave 90% specific $^{51}$Cr release in the absence of antibody (Table I).

Inhibition of CTL-mediated killing by M7/14 follows a typical dose-response pattern and is quite potent (Fig. 2). Half-maximal inhibition occurs at a final dilution of MAbCS of 1/150, corresponding to 600 ng IgG/ml.

M7/14 MAb not only inhibits xenogeneic anti-BNLφ CTL-mediated lysis, but all CTL thus far tested. These include 3 different murine allogeneic systems and an anti-modified self syngeneic system (Table II). Inhibition in these systems is as good as or often better than that observed for Lyt-2 MAb (unpublished data). M7/14 does not appear to recognize an alloantigenic determinant, as shown by the above findings. Furthermore, M7/14 is positive on 9 of 9 mouse strains tested in the indirect binding assay, i.e., A/J, AKR, BALB/c, B10.A, B10.A(5R), B10.BR, B6, DBA/1, and SJL.

*Blocking by M7/14-Ig is not an artifact of agglutination.* Efficient T cell-mediated killing requires intimate contact between CTL and target cells (19). This is promoted by vortex mixing followed by centrifugation, and probably also by CTL motility during incubation. Agglutination of CTL during pretreatment with antibody would be a trivial means of inhibiting killing, and this possibility was therefore tested (Table III). It was found that M5/49 supernatant (anti-Thy-1) gave about 40% agglutination of sensitized lymphocytes (doubly underlined values), but did not inhibit killing. In contrast, M7/14 supernatant inhibited killing strongly, but produced little or no agglutination. These results demonstrate that the inhibition of killing produced by M7/14 supernatants is not due to agglutination.

*M7/14 blocks CTL-target conjugate formation.* In the above experiment, conjugates between sensitized lymphocytes and target cells formed in the absence of antibody in a rapid (5 min), temperature-dependent (37°C) manner (Table III), as previously described (19). Conjugate formation was inhibited by M7/14 (Table III, singly underlined value). In a 2nd representative experiment, the formation of adhesive CTL-target cell conjugates was inhibited

TABLE I

*Purified M7/14 immunoglobulin blocks T lymphocyte-mediated cytolysis*[a]

| Expt. No. | E:T Ratio | % Corrected $^{51}$Cr Release | | | % Inhibition by M7/14 |
|---|---|---|---|---|---|
| | | No addition | Normal rat IgG[b] | M7/14 IgG[b] | |
| 1 | 60:1 | 33 ± 4.6 | 31.6 ± 3.4 | 1.8 ± 1.7 | 94.5 |
| 2 | 60:1 | 93.3 ± 3.1 | 88.8 ± 3.2 | 9.4 ± 0.2 | 90 |
| 2 | 6:1 | 41.3 ± 3.5 | 40.6 ± 0.8 | 3.3 ± 2.8 | 92 |

[a] Effector cells were C57BL/6 anti-rat BNLφ; targets were $^{51}$Cr-labeled BNLφ. Spontaneous $^{51}$Cr release was 10 to 15%. Percentage corrected release values represent the mean ± SE of duplicate observations (triplicate observations for tubes containing no immunoglobulin).

[b] Effector cells were pretreated with 6.25 μg of purified Ig in a volume of 0.1 ml, then an equal volume of target cells was added.

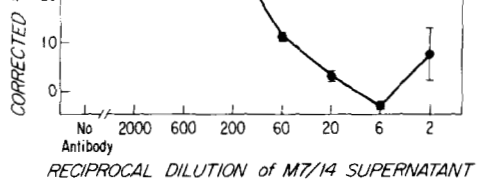


*Figure 2.* Dose response for inhibition by M7/14 supernatant of C57BL/6 anti-BN lymphoma killing. Sensitized/target cell ratio was 20, and final incubation was for 2 hr at 37°C. Spontaneous $^{51}$Cr release was 12 to 14.5%. Points represent the mean ± SEM for duplicate determinations.

TABLE II

*M7/14 blocks killing directed to allogeneic and modified-self determinants*[a]

| Expt. No. | Effector Cell | Target | E:T Ratio | % Corrected $^{51}$Cr Release | | % Inhibition |
|---|---|---|---|---|---|---|
| | | | | −M7/14 | +M7/14 | |
| 1 | C57BL/6 | P815 | 1.5:1 | 45 | 15 | 66 |
| 2 | C57BL/6 | P815 | 1.1:1 | 43 | 9 | 78 |
| 3 | AKR/J | P815 | 1.7:1 | 52 | 27 | 48 |
| 4 | CD2F$_1$ | EL-4 | 2:1 | 37 | 10 | 73 |
| 5 | CD2F$_1$ | TNP-P815 | 60:1 | 27 | −0.5 | 100 |

[a] Final incubation was 4 hr at 37°C. Spontaneous $^{51}$Cr release was 9 to 22%. Immunizing cells were the same as target cells except for expt. 5, TNP-splenocytes from (BALB/c × DBA/2)F$_1$ mice.

72% after 21 min at 37°C and was highly statistically significant (Table IV).

*M7/14-Ig blocks a variety of T cell functions: The mixed lymphocyte response (MLR).* The effects of M7/14 on a number of other T cell functions were also tested. In analogy to the xenogeneic CTL system described above, a xenogeneic B6 mouse anti-LBN rat MLR system was used to exclude formally the possibility of MAb binding to target cell antigens. However, allogeneic MLR systems were also utilized in many experiments and gave higher [$^3$H] thymidine incorporation. In all 5 of 5 independent experiments carried out on different days, both M7/14 culture supernatants and pure IgG inhibited the allogeneic MLR an average of 86%, SD = 8% (Fig. 3). In 4 of 4 xenogeneic experiments, average inhibition was 95%, SD = 12% (Fig. 3). Supernatant from the M1/87 anti-Forssman line, which does not label splenic lymphocytes (15), demonstrated no nonspecific effect by spent culture supernatant. MAb to H-2 and Thy-1 were also without inhibitory effect (Fig. 3). This suggests that inhibition of the MLR by M7/14 is not due to antibody bound to the cell surface *per se*, but depends on the blockade of a specific cell surface site. Partial inhibition of allo but not xeno MLR cultures by the M7/81 anti-Ia MAb (Fig. 3) could be due to blocking of allostimulation determinants.

Dose-response characteristics of MLR inhibition were studied for both M7/14 culture supernatants and purified IgG (Fig. 4). Inhibition was maximal at 25 to 2.5 μg/ml of antibody, and was still greater than 50% at 0.25 μg/ml ($1.5 \times 10^{-9}$ M), corresponding to a 400-fold dilution of the culture supernatant. The similar dose-response characteristics of M7/14 culture supernatants and pure IgG shows that inhibition is mediated by IgG rather than other factors secreted by M7/14 hybrid cells.

The effect of time of addition of M7/14 to the MLR was also studied (Table V). Addition on day 0 but not on days 1, 2, or 4 of the 6-day culture period gave inhibition. This shows that M7/14 blocks at an early stage in the MLR proliferative response and is not toxic to MLR cultures.

The same antibodies that were used in Figure 3 were also tested for inhibition of Con A responses (data not shown). Only M7/14 gave some inhibition of Con A stimulation (mean = 42%), which was found to occur in a dose-dependent manner, but it was highly variable from experiment to experiment (SD = 28%).

*CTL generation in vitro.* Secondary generation of CTL *in vitro* (B6 anti-BNLφ, see *Materials and Methods*) was inhibited by purified M7/14 Ig in 2 of 2 experiments (data not shown). Clear-cut, consistent inhibition appeared to require more than 2 μg Ig/ml. Inhibition was about 5-fold in 1 experiment and virtually complete in the 2nd experiment (>>10-fold).

*Antigen-specific T cell proliferation.* Inhibition of the antigen-specific proliferative response to a soluble antigen, GAT,[5] was also tested. The response was consistently blocked by M7/14 supernatant but not by anti-Thy-1 MAb (M5/49, M5/56) or another MAb binding to activated T cells (M5/78) (representative experiment shown in Table VI).

*M7/14 MAb inhibits T-dependent but not T-independent B cell responses: PFC (Jerne plaque assay) responses.* Primary antibody responses by mouse splenocytes in Mishell-Dutton cultures to T-dependent (SRBC) and T-independent (NP$^5$-Ficoll) antigens were studied using procedures described elsewhere (20). The results in 3 of 3 experiments (Table VII) showed inhibition of T-dependent but not T-independent PFC responses by M7/14 antibody. The inhibition was seen with purified M7/14-IgG, but not with similarly purified normal rat IgG. MAb of the same IgG2a subclass to the

TABLE III

*Inhibition of CTL-mediated cytolysis by M7/14 is not an artifact of agglutination*[a]

| Antibody | Centrifugation | Incubation | Lymphocytes: % Small cells (lymphocytes) agglutinated[b] | Lymphocytes Plus Targets: % Small cells (lymphocytes) agglutinated[b] | Lymphocytes Plus Targets: % Large cells (targets) in conjugates[c] | Corrected % $^{51}$Cr released[d] (% Inhibition) |
|---|---|---|---|---|---|---|
| None | − | None | 3 | 1 | 5 | |
| | + | 0°C | 6 | 4 | 4 | |
| | + | 37°C, 5 min | 6 | 1 | 25 | |
| | + | 37°C, 2 hr | | | | 77% |
| M7/14 | − | None | 0 | 0 | 4 | |
| (Anti-LFA-1) | + | 0°C | 5 | 3 | 9 | |
| | + | 37°C, 5 min | 1 | 0 | 5 | |
| | + | 37°C, 2 hr | | | | 6% (92%) |
| M5/49 | − | None | 1 | 2 | 8 | |
| (Anti-Thy-1) | + | 0°C | 51 | 38 | 7 | |
| | + | 37°C, 5 min | 42 | 47 | 10 | |
| | + | 37°C, 2 hr | | | | 77% (0%) |

[a] C57BL/6 anti-BNL$\phi$-sensitized cells were pretreated with M7/14 or M5/49 supernatants or sham pretreated with medium for 30 min at room temperature (vortexing at 0 min and 15 min). On ice, either medium or 5 × $10^4$ $^{51}$Cr-labeled BNL$\phi$ cells were added to give lymphocyte/target cell ratios of 10. The final volume was 0.15 ml. Microscopic observations were made immediately (no centrifugation) or after centrifugation at 4°C for 5 min at 500 × G after which the tubes were either placed on ice or incubated at 37°C for 5 min before scoring. Each tube was vortexed immediately before scoring. Some tubes were incubated at 37°C for 2 hr at which time the amount of $^{51}$Cr release was determined.

[b] The percentage of small cells (sensitized lymphocytes) that were agglutinated to other small cells is expressed as 100 × (total number of cells involved in clusters of two or more)/(total number of cells counted). In all cases greater than 100 cells were counted.

[c] The percentage of large cells (targets) in conjugates is expressed as 100 × (number of large cells adhering to one or more small cells)/(total number of large cells counted). At least 100 targets were counted for each procedure. In this experiment, 2.1% of the lymphocyte population was large enough to be confused with target cells.

[d] The spontaneous $^{51}$Cr release in tubes from which effectors were omitted was 8 to 11%.

TABLE IV

*M7/14 inhibits effector-target conjugate formation*[a]

| Min at 37°C | M7/14 | % of Targets in Conjugates | $P_{X^2}$ |
|---|---|---|---|
| 0 | − | 3.7 | |
| 21 | − | 44.3 | |
| 21 | + | 15.0 | <0.001 |

[a] Six × $10^5$ C57BL/6 anti-BNL$\phi$ lymphocytes were pretreated with medium or purified M7/14 immunoglobulin (3.12 $\mu$g in a volume of 0.05 ml) for 15 min at room temperature. On ice, 5 × $10^4$ $^{51}$Cr-labeled BNL$\phi$ cells in 0.025 ml were added. The tubes were vortexed, centrifuged at 4°C for 5 min at 500 × G, and then incubated at 37°C for the time indicated. (The 21 min incubation consisted of 3 individual 7 min incubations separated by vortexing and centrifugation.) Tubes were then vortexed twice for 2 sec at setting 2, and were microscopically scored for the percentage of large cells adhering to 1 or more small cells (% of targets in conjugates). In this experiment, 1.1% of the effector population were large enough to be confused with target cells.

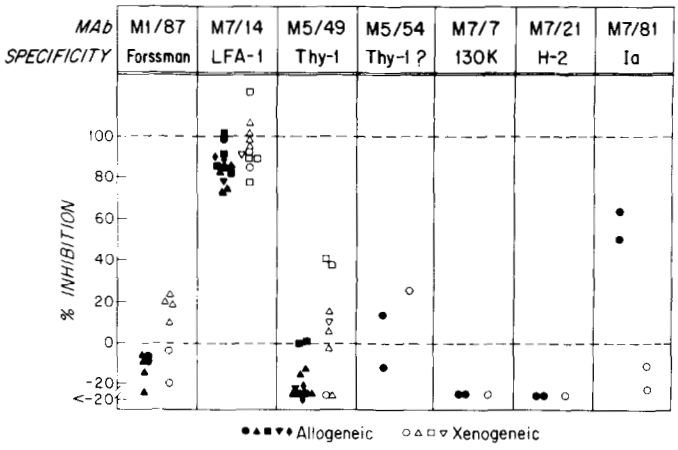


*Figure 3.* Inhibition by monoclonal antibodies of the allo- and xenogeneic MLR. Monoclonal antibodies secreted into tissue culture medium or as the purified IgG were added at the initiation of B6 anti-BALB/c (*allogeneic*), B6 anti-(L × BN)$F_1$ rat (*xenogeneic*) or B6 anti-B6 syngeneic (*control*) MLR cultures. Concentrations ranged from 2 to 25% for M7/14 supernatants, and 2.5 to 25 $\mu$g/ml for pure M7/14 IgG. All inhibition experiments have been included, and different symbol shapes represent independent experiments conducted on different days. Specific [$^3$H] thymidine incorporation on day 5 was calculated by subtracting the syngeneic control, and % inhibition was expressed relative to cultures receiving normal medium.

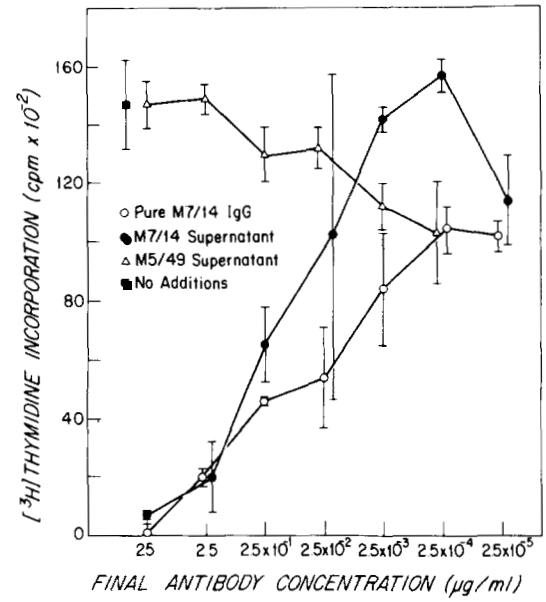


*Figure 4.* Dose response for M7/14 IgG and MAbCS in MLR inhibition. Purified M7/14 IgG dialyzed *vs* culture medium, M7/14 MAbCS (94 $\mu$g IgG/ml), or M5/49 MAbCS (87 $\mu$g/ml) were added at the indicated concentration at the initiation of B6 anti-BALB/c or syngeneic control MLR cultures. The data are expressed as specific thymidine incorporation, after subtraction of the syngeneic control. Bars show the SEM.

common leukocyte antigen (M1/9.3) or H-2 (M1/42) did not inhibit.

*LPS-stimulated B cell responses.* M7/14 had no inhibitory effect on LPS-stimulated B cell mitogenesis (a representative experiment is shown in Table VIII). In 5 of 5 independent experiments, LPS blastogenesis was, if anything, slightly stimulated by M7/14 (mean stimulation = 13%, SD = 26%).

## DISCUSSION

Previously, we obtained and screened a large panel of rat anti-mouse MAb for their effect on xenogeneic mouse CTL-mediated lysis of rat target cells (1). One MAb, M7/14, was of particular interest because it strongly inhibited CTL-mediated killing and defined a novel lymphocyte surface antigen containing polypeptide

TABLE V

*Time course of inhibition of the MLR*[a]

| Day of Culture | Addition[c] | $^3$H-Thymidine[b] cpm × $10^{-2}$ | % Inhibition |
|---|---|---|---|
| 0 | None | 115 | (0) |
| | M5/49 | 142 | 0 |
| | M7/14 | 25 | 78 |
| 1 | None | 123 | (0) |
| | M5/49 | 100 | 19 |
| | M7/14 | 106 | 14 |
| 2 | None | 83 | (0) |
| | M5/49 | 116 | 0 |
| | M7/14 | 132 | 0 |
| 4 | None | 125 | (0) |
| | M5/49 | 154 | 0 |
| | M7/14 | 138 | 0 |

[a] On the appropriate day, 50 μl of culture medium was replaced with 50 μl of MabCS diluted 1:10 in culture medium, or with medium only (no addition).
[b] (B6 anti-BALB/c) − (B6 anti-B6) incorporation.

TABLE VI

*M7/14 blocking of GAT-specific T cell proliferation*[a]

| MAb[b] | Δcpm[c] | % Inhibition[d] |
|---|---|---|
| None | 81,579 | |
| M5/49 | 90,047 | 0 |
| M5/78 | 72,058 | 12 |
| M5/56 | 84,038 | 0 |
| M7/14 | 3,379 | 96 |

[a] Mice were primed with GAT in complete Freund's adjuvant. After 7 days, primed nylon wool purified T cells were prepared and cultured with GAT ± MAbCS for 3 days. The cultures were then pulsed with tritiated thymidine for 18 hr and harvested to measure thymidine incorporation (details of this procedure are in 31).
[b] Thirty percent final concentration of original hybridoma culture supernatant.
[c] Δcpm = (cpm of responding T cells + MAb + GAT) − (cpm of responding T cells + MAb only).
[d] Controls in which MAb was omitted were not significantly different from NSI supernatant controls.

chains of 180,000 and 95,000 $M_r$. The antigen was designated LFA-1, and findings suggested that it participates in or is closely associated with the multi-step functional pathway involved in cell-mediated killing. To date, only 2 CTL cell surface components, Lyt-2,3 (1, 4–9) and LFA-1 (1) have been shown to be susceptible to antibody-mediated blockade of CTL function.

In the present study we have examined in more detail the effect of M7/14 MAb on CTL function and have also studied the effect on a broad range of T and B lymphocyte functional responses. M7/14 MAb had previously been shown to inhibit xenogeneic CTL-mediated killing, and was shown here also to inhibit allogeneic and anti-modified self CTL-mediated killing.

Inhibition of functional responses was clearly due to the specific antibody secreted by M7/14, since 1) purified M7/14 IgG gave the same level of inhibition of CTL-mediated killing as previously shown for M7/14 MAbCS, 2) MAbCS and purified IgG had similar dose-response characteristics in MLR inhibition, and 3) inhibition of CTL-mediated killing, the MLR, and antigen-specific proliferation was not given by other MAbCS.

Trivial means of inhibition were investigated. M7/14 does not agglutinate CTL, i.e., sensitized lymphocytes. In contrast, the M5/49 anti-Thy-1 MAb gave strong agglutination of sensitized lymphocytes, but gave little or no inhibition of killing. The viability of CTL preparations has been measured by trypan blue exclusion at the end of the CTL assay, and found to be unimpaired by M7/14 MAb (unpublished data). Thus inhibition of CTL-mediated killing by trivial means such as agglutination, toxicity, or binding to the target cell (1) have been ruled out.

CTL-mediated killing has previously been shown to involve at least 2 steps, recognition-adhesion and lethal hit delivery, which are separable physiologically and pharmacologically (reviewed in 19). Tight adhesions between CTL and target cells are formed in 0.5 to 5 min at 37°C in a process that is dependent on specific antigen recognition and $Mg^{+2}$. In a 2nd step, which is $Ca^{+2}$ dependent, the lethal hit is delivered in 5 to 15 min at 37°C. After this, CTL can be detached from target cells, and killer cell-independent lysis proceeds, releasing $^{51}$Cr in 0.5 to 2 hr. M7/14 MAb blocks the recognition-adhesion step, as shown by inhibition of CTL-target cell conjugate formation. MAb to Lyt-2,3 have also been reported to block this step (6). There is evidence that the recognition-adhesion step may be further separable into crawling or surface motility to engage the target (21–24), specific antigen recognition (25), and adhesion (12, 22). We have preliminary evidence (unpublished) that M7/14 does not inhibit "crawling," i.e., the ability of lymphocytes to adhere to and crawl under fibroblast monolayers (21). LFA-1 is probably not identical to the T cell antigen receptor, since it is expressed on non-T lymphocytes, including B lymphcytes and myeloid cells (26). Thus, it is our working hypothesis that LFA-1 is a protein distinct from the antigen receptor that is important in the adhesion process.

The effect of M7/14 MAb was also studied on a number of other T cell functional responses. MLR proliferation was 95% inhibited in a xenogeneic system analogous to the CTL system described above in which binding to stimulator cells can be ruled out by the species combinations. Allogeneic MLR responses were also inhibited by 86%. The antigen-specific proliferative response to GAT *in vitro* was inhibited by 96%. The findings that M7/14 MAb had no effect when added at day 1 in 6-day MLR cultures, and that antibodies to H-2 and Thy-1 had no effect on MLR, proliferation to antigen, or T-dependent B cell responses, show that M7/14 and other antibodies of the same subclass bound to the surface were not toxic to the cells in culture. These findings suggest that LFA-1 participates in or is closely associated with a large number of T cell functional responses.

Previously, monoclonal antibodies to 2 other types of antigens

TABLE VII

*Selective blocking of T dependent PFC responses by M7/14*[a]

| | Ag | MAb | PFC/culture | % Inhibition |
|---|---|---|---|---|
| Expt. 1 | SRBC | None | 3690 | |
| | SRBC | 25% M7/14 | 1860 | 50 |
| | SRBC | 10% M7/14 | 2280 | 38 |
| | NP-Ficoll | None | 2430 | |
| | NP-Ficoll | 25% M7/14 | 3930 | 0 |
| | NP-Ficoll | 10% M7/14 | 2550 | 0 |
| Expt. 2 | SRBC | None | 2265 | |
| | SRBC | 25% M7/14 | 705 | 69 |
| | SRBC | 10% M7/14 | 225 | 90 |
| | NP-Ficoll | None | 2700 | |
| | NP-Ficoll | 25% M7/14 | 2865 | 0 |
| | NP-Ficoll | 10% M7/14 | 2790 | 0 |
| Expt. 3 | SRBC | None | 2660 | |
| | SRBC | Rat IgG, 100 μg/ml | 2380 | 11 |
| | SRBC | Pure M7/14, 100 μg/ml | 740 | 72 |
| | SRBC | 25% M7/14 | 1180 | 56 |
| | SRBC | 25% M1/9.3 | 2440 | 8 |
| | SRBC | 25% M1/42 | 3900 | 0 |
| | NP-Ficoll | None | 1360 | |
| | NP-Ficoll | Rat IgG, 100 μg/ml | 1320 | 3 |
| | NP-Ficoll | Pure M7/14, 100 μg/ml | 1200 | 12 |
| | NP-Ficoll | 25% M7/14 | 2320 | 0 |
| | NP-Ficoll | 25% M1/9.3 | 1220 | 10 |
| | NP-Ficoll | 25% M1/42 | 1200 | 12 |

[a] Methods are detailed in Zubler *et al.* (20). NP-Ficoll was prepared by coupling amino-ethyl-carboxy-methyl-ficoll (Biosearch, Inc., San Rafael, CA) with the reactive NP-O-Succinimide (Biosearch, Inc.) to yield a conjugate containing ~42 NP groups/100,000 m.w. of ficoll. After extensive dialysis, this material was filtered through a 0.45 μ Millex filter and stored at 4°C until use at 0.1 μg/ml in the cultures. NP-SRBC were prepared by adding 2.5 mg NP-O-Succinimide in 0.2 ml dimethylformanide to 10 ml of 10% washed SRBC in 0.2 M $NaHCO_3$, pH 9.0. After 10 min at room temperature, the cells were washed 3 times with PBS, and used in the Jerne plaque assay.

TABLE VIII

*M7/14 does not inhibit LPS-stimulated B cell mitogenesis*

| | Additions | $^3$H-thymidine Incorporation ± SD (cpm × $10^{-3}$) |
|---|---|---|
| −LPS | | 3.5 ± 0.3 |
| +LPS | | 65 ± 14 |
| +LPS | 25% M7/14 MAbCS | 70 ± 6 |
| +LPS | 5% M7/14 MAbCS | 77 ± 4 |

have been found to inhibit the MLR in cases where reactivity with stimulator cells was excluded. Anti-Lyt-2,3 antibodies inhibit (7, 27), but the inhibition is less complete than that seen with M7/14. A monoclonal antibody that is specific for the helper T cell subset in the rat, W3/25, potently inhibits the MLR (28). This was shown not to involve blockade of antigen on stimulator cells by using a sorted subpopulation of $W3/25^-$ cells as stimulators.

The effect of M7/14 on both T-dependent and T-independent *in vitro* PFC responses was also studied. The T-independent PFC response to TNP-Ficoll was not inhibited by M7/14. Nor did M7/14 have any inhibitory effect on LPS-stimulated B cell mitogenesis. The T-dependent PFC response was inhibited by a mean of 63% by M7/14. The findings with LPS and TNP-Ficoll suggest M7/14 has no effect on those B cell responses that are independent of cellular interactions.

It is tempting to speculate that in all the functional responses inhibited by M7/14 MAb, LFA-1 participates in forming adhesions required for cellular interactions. The evidence for this has already been discussed in the case of CTL adhering to target cells. For T cell responses in the MLR, proliferation to antigen, and T-dependent B cell responses, T cell-macrophage interactions are required (29). LFA-1 could participate in an adhesion step closely linked to the major histocompatibility complex-restricted interaction, i.e., recognition by CTL of H-2 on target cells and recognition by the T helper cell of Ia on macrophages (30). Inhibition of the MLR by M7/14 on day 0 but not on day 1 would be consistent with blockade of an early recognition-adhesion event. No cellular interactions are thought to be required for B cell responses to LPS or TNP-Ficoll. This may explain the lack of effect of M7/14 on these responses, despite the fact that B cells express LFA-1 (26).

*Acknowledgments.* We thank Leonard Colarusso and Evelyn Reilly for skillful technical assistance. We are grateful to Dr. Ronald N. Germain for the data in Tables VI and VII. We appreciate word processing by Harriet Yake and Terri Greenberg.

## REFERENCES

1. 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. In press.
2. 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.
3. Ledbetter, J. A., and L. A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. 47:63.
4. 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.
5. 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. 76:1977.
6. 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 -nonspecific cytotoxicity: evidence for the blocking of the binding between T lymphocytes and target cells and not the post-binding cytolytic steps. J. Immunol. 125:2444.
7. Hollander, N., E. Pillemer, and I. L. Weissman. 1980. The blocking effect of Lyt-2 antibodies on T cell functions. J. Exp. Med. 152:674.
8. 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.
9. 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.
10. Dunn, T. B., and M. Potter. 1957. A transplantable mast-cell neoplasm in the mouse. J. Natl. Cancer Inst. 18:587.
11. Gorer, P. A. 1950. Studies in antibody response of mice to tumour inoculation. Br. J. Cancer 4:372.
12. Martz, E. 1980. Immune lymphocyte to tumor cell adhesion: magnesium sufficient, calcium insufficient. J. Cell Biol. 84:584.
13. Burakoff, S. J., R. N. Germain, M. E. Dorf, and B. Benacerraf. 1976. Inhibition of cell-mediated cytolysis of trinitrophenyl-derivitized target cells by alloantisera directed to the products of the K and D loci of the H-2 complex. Proc. Natl. Acad. Sci. 73:625.
14. Parker, W. L., and E. Martz. 1980. Lectin-induced nonlethal adhesions between cytolytic T lymphocytes and antigenically unrecognizable tumor cells, and nonspecific "triggering" of cytolysis. J. Immunol. 124:25.
15. Springer, T. A. 1980. Cell-surface differentiation in the mouse. Characterization of "jumping" and "lineage" antigens using xenogeneic rat monoclonal antibodies. *In* Monoclonal Antibodies, Edited by R. H. Kennett, T. J. McKearn, and K. B. Bechtol. Plenum, New York. Pp. 185–217.
16. Meo, T. 1979. The MLR test in the mouse. *In* Immunological Methods, Edited by I. Lefkovits and B. Pernis. Academic Press, New York. Pp. 227.
17. Ault, K. A., and T. A. Springer. 1980. Cross-reaction of a rat-anti-mouse phagocyte-specific monoclonal antibody (anti-Mac-1) with human monocytes and natural killer cells. J. Immunol. 126:359.
18. Cotton, R. G. H., D. S. Secher, and C. Milstein. 1973. Somatic mutation and the origin of antibody diversity. Clonal variation of the immunoglobulin produced by MOPC 21 cells in culture. Eur. J. Immunol. 3:135.
19. 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.
20. Zubler, R. H., H. Cantor, B. Benacerraf, and R. N. Germain. 1980. Feedback suppression of the immune response *in vitro*. I. Activity of antigen-stimulated B cells. J. Exp. Med. 151:667.
21. Chang, T. W., E. Celis, H. N. Eisen, and F. Solomon. 1979. Crawling movements of lymphocytes on and beneath fibroblasts in culture. Proc. Natl. Acad. Sci. 76:2917.
22. Gately, M. K., W. J. Wechter, and E. Martz. 1980. Early steps in specific tumor cell lysis by sensitized mouse T lymphocytes. IV. Inhibition of programming for lysis by pharmacologic agents. J. Immunol. 125:783.
23. Rothstein, T. L., M. Mage, G. Jones, and L. L. McHugh. 1978. Cytotoxic T lymphocyte sequential killing of immobilized allogeneic tumor target cells measured by time-lapse microcinematography. J. Immunol. 121:1652.
24. Sanderson, C. J. 1976. The mechanism of T cell-mediated cytotoxicity. II. Morphological studies of cell death by time-lapse microcinematography. Proc. R. Soc. Lond. B 192:241.
25. Shortman, K., and P. Golstein. 1979. Target cell recognition by cytolytic T cells: different requirements for the formation of strong conjugates or for proceeding to lysis. J. Immunol. 123:833.
26. 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.
27. Nakayama, E., W. Dippold, H. Shiku, H. F. Oettgen, and L. J. Old. 1980. Alloantigen-induced T-cell proliferation: Lyt phenotype of responding cells and blocking of proliferation by Lyt antisera. Proc. Natl. Acad. Sci. 77:2890.
28. Mason, D. W., R. J. Brideau, W. R. McMaster, M. Webb, R. A. H. White, and A. F. Williams. 1980. Monoclonal antibodies that define T-lymphocyte subsets in the rat. *In* Monoclonal Antibodies, Edited by R. H. Kennett, T. J. McKearn, and K. B. Bechtol. Plenum, New York. Pp. 251–273.
29. Benacerraf, B. 1981. The role of MHC gene products in immune regulation and its relevance to alloreactivity. Science 212:1229.
30. 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.
31. Letvin, N., J. T. Nepom, M. I. Greene, B. Benacerraf, and R. N. Germain. 1980. Loss of Ia bearing splenic adherent cells after whole body ultraviolet irradiation. J. Immunol. 125:1402.
32. Laemmli, U. K., and M. Favre. 1973. Maturation of the head of bacteriophage T4. J. Mol. Biol. 80:575.
33. Vesterberg, O. 1971. Staining of protein zones after isoelectric focusing in polyacrylamide gels. Biochim. Biophys. Acta 243:345.