Brief Definitive Report

MODIFICATION OF MURINE T-CELL CYTOTOXICITY BY PREIMMUNIZATION WITH *M* LOCUS AND *H-2* INCOMPATIBILITIES

By A. MATOSSIAN-ROGERS and H. FESTENSTEIN

(From the Tissue Immunology Unit, London Hospital Medical College, London, E1.2AD)

Two main methods have been used in the in vitro study of allograft rejection: mixed lymphocyte cultures (MLC) and cell-mediated lympholysis (CML). Because the proliferative phase in MLC does not necessarily provoke the generation of cytotoxic T lymphocytes (1), the CML assay is considered to be a better model of allograft rejection. The generation of cytotoxic effector cells is dependent on major histocompatibility system (MHS) disparity between effector and target cells (2, 3). Some sensitive CML assays have recently enabled the detection of cytotoxic T cells activated against the H-2 antigenic specificities alone (4); however, amplification of CML by added *I*-region disparity has been noted (2, 3, 5). The *I* region contains lymphocyte-activating determinants (LADs) to which amplification has been attributed and LADs outside the MHS (6–8) have also been claimed to substitute for *I*-region LADs (9, 10).

MLC tests are enhanced by preimmunization of donor animals across MHS barriers (11, 12), but are suppressed when preimmunized with *M*-locus incompatibility (6, 13).

The aim of the present experiments is to examine the effects of *H-2* and *M*-locus preimmunization on the generation in MLC of effector cells assayed by CML.

Materials and Methods

Mice. Adult female mice of the following inbred strains and F_1 hybrids were used: CBA/H (*H-2*^{k},*Mls*^{b}), BRVR (*H-2*^{k},*Mls*^{a}), BALB/c (*H-2*^{d},*Mls*^{b}), DBA/2 (*H-2*^{d},*Mls*^{a}), and (CBA/H × DBA/2)F_1 mice.

Experimental Design. Groups of CBA/H mice were immunized intraperitoneally with 10^7 spleen cells from (*a*) BRVR (*M*-locus incompatible), (*b*) BALB/c (*H-2* incompatible), and (*c*) syngeneic cells. For certain experiments, groups (*a*) and (*b*) were given a second injection on day 6. At various times after immunization lymph node cells were removed from mice in each of the immunized groups and cultured with (CBA/H × DBA/2)F_1 lymphocytes. The generation of cytotoxic effector cells after a 5-day sensitization period was tested against phytohemagglutinin (PHA) transformed DBA/2, BRVR, and CBA/H as well as LPS-transformed BRVR lymphocytes.

Mixed Lymphocyte Cultures. Axillary lymph nodes were removed from the preimmunized mice and from normal (CBA/H × DBA/2)F_1 mice, gently pushed through metal sieves of fine mesh and washed twice in Eagle's minimal essential medium (Wellcome Research Laboratories, Kent, England). The cells were then resuspended at a concentration of 5×10^6/ml in RPMI (Grand Island Biological Co., Grand Island, N. Y.) medium containing glutamine and supplemented with antibiotics, 10 mM HEPES, 10% fetal calf serum, and 5×10^{-5} M 2-mercaptoethanol. The responder and stimulator cell suspensions were mixed in equal proportion and dispensed in 200 μl volumes into flat-bottomed wells of Cooke microtest II plates (Cooke M220-29AR, Cooke Labora-

tory Products, Alexandria, Va.). The plates were incubated at 36°C in partially evacuated containers which were gassed with a mixture of 92% N_2 and 8% CO_2, leaving a residual vacuum of 100 mmHg and a gas mixture 86% N_2, 10% O_2, and 4% CO_2.

Cell-Mediated Lymphocytotoxicity Assay (CML). The assays were carried out as previously described (14). Briefly, effector cells from 5th day MLC were pooled and washed once before the assay. Target cells cultured for 2–3 days in the presnce of 1 μg/ml PHA (Wellcome Research Laboratories) were washed once and labeled with 100 μCi ^{51}Cr-sodium chromate, washed twice after labeling, and the viable blasts reconstituted to 4×10^5 cells per ml. The target cell suspensions were dispensed in 25-μl amounts into wells of Cooke microtest II plates; immune and normal effector cells were added in 200 μl volumes to the target cells in proportions of 20:1, 40:1, and 80:1 with triplicates at each ratio. To estimate maximum release 200 μl distilled water were added to the target cells. The plates were lightly spun for 5 min and incubated for 3 h at 36°C in a CO_2 atmosphere. At the end of the incubation period 50 μl of supernate were removed from each well for counting. Specific release of isotope was calculated according to the following formula: 100 × [cpm (immune effectors) − cpm (normal effectors)]/[maximum release − cpm (normal effectors)].

Results

Effect of Preimmunization on the Generation of Cytotoxic Lymphocytes against H-2 Targets. The effect of preimmunization of CBA/H mice with one injection of BRVR and BALB/c spleen cells on the subsequent generation in vitro of effector cells against DBA/2 is shown in Table I. Sensitization in MLC initiated 3 days after immunization resulted in enhanced cytotoxic activity against DBA/2 when the responder cells were obtained from either BALB/c (*H-2* incompatible) or BRVR (*M*-locus incompatible) immunized groups; the specific ^{51}Cr release was approximately twice (2.39 and 1.96 times, respectively) that produced by effectors derived from syngeneic immunized control mice. When lymphocytes were removed for in vitro sensitization 6 days after immunization, effectors generated from the BALB/c immunized group retained their enhanced lytic activity compared to the control group. Lymphocytes obtained from the BRVR immunized group, however, showed suppressed lytic activity on DBA/2 targets.

Suppression of the cytotoxic response reached a maximum on the 15th day after immunization and thereafter waned gradually. By the 28th day, enhancement of cytotoxicity was noted in both immunized groups. The enhanced cytotoxic activity of the BALB/c immunized mice, noted 3 days after immunization, was relatively suppressed from the 6th to the 15th day, as shown in Fig. 1 and Table I, but a net increase in lytic activity compared to the control group was maintained throughout the experimental period.

A second injection of 10^7 spleen cells given 6 days after the first injection resulted in abrogation of cytotoxicity to DBA/2 targets in the *M*-locus-immunized mice tested on the 15th day after the beginning of the immunization schedule (Table II). The small percentage specific release of isotope (3.8%) was found not to represent significant kill by an analysis of variance test. Suppression of cytotoxic response after the second injection was maintained for up to 60 days after the beginning of immunization.

Effect of Preimmunization on the Generation of Cytotoxic T Lymphocytes against M-locus Incompatible Targets. As we were previously unsuccessful in generating effector cells in MLC against *M*-locus incompatible target cells, we tried to see if preimmunization would make any difference. Lymphocytes were

TABLE I

Cytotoxicity of Effectors from CBA/H Mice Preimmunized with Syngeneic, M Locus, and H-2-Incompatible Cells and Cultured in vitro with (CBA/H × DBA/2)F_1 Cells

Cells for in vitro sensitization taken from CBA/H mice immunized against:	Lymphocytes removed from mice for MLC on days indicated after preimmunization of mice:				
	3	6	15	19	28
	% specific ^{51}Cr released from DBA/2 targets				
CBA/H	15.2 (1)*	20.6 (1)	20.1 (1)	28.7 (1)	17.1 (1)
BRVR	29.8 (1.96)	11.1 (0.53)	8.8 (0.44)	19.3 (0.67)	22.6 (1.32)
BALB/c	36.1 (2.37)	29.2 (1.41)	28.7 (1.42)	54.6 (1.89)	62.7 (3.66)

Differences in specific ^{51}Cr release between the control and test immunized groups were significant within the range $P < 0.025$ to $P < 0.001$ estimated by analysis of variance tests.

Nonspecific kill estimated by isotope release from CBA/H targets in all experiments fell in the range 0.02–1.65%.

* The percent ^{51}Cr release figures were transformed by expressing them relative to the syngeneic control which thus became unity.

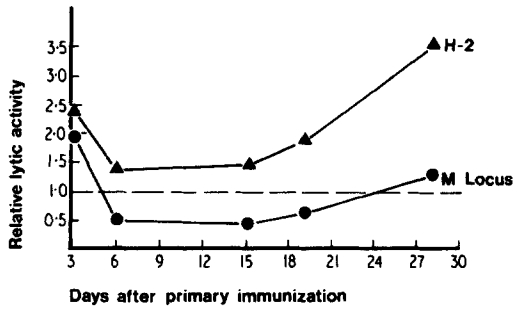

FIG. 1. Time-course study of the anti-H-2 cytotoxic behavior of lymphocytes from CBA/H mice immunized with *H-2*-incompatible (▲), *M*-locus incompatible (●), and syngeneic cells (– –) and sensitized in vitro against (CBA/H × DBA/2)F_1 cells.

removed from preimmunized mice for in vitro sensitization with (CBA/H × DBA/2)F_1 cells at various intervals after immunization, and cytotoxic effectors against BRVR targets were assayed. Representative results are shown in Table III. Sensitization of syngeneically immunized CBA/H mice against *M*-locus disparate cells in presence of added *H-2* incompatibility did not generate effectors against *M*-locus targets. Similarly, preimmunization of CBA/H mice with *M*-locus or *H-2* incompatibilities was equally ineffective in providing a boost for the generation in vitro of effectors against *M*-locus targets. This may indicate that clones reactive to *M*-locus and *H-2* LADs are distinct, and there is no co-operation between such activated clones.

Discussion

Preimmunization of CBA/H mice with *M*-locus incompatible cells did not help to generate cytotoxic effectors in MLR against the same M-antigen-bearing targets. The anti-*H-2* response of *M*-locus preimmunized mice, however, was characterized by a short period of amplification followed by a suppressed phase (Fig. 1). It has previously been shown that preimmunization against *M* locus produced a lower response in MLR 12 days after spleen cell injections (6), a

TABLE II

Cytotoxicity of Effectors from CBA/H Mice Preimmunized and Boosted on Day 6 with BRVR and BALB/c Cells and Subsequently Cultured In Vitro with (CBA/H × DBA/2)F_1 Cells

Cells for in vitro sensitization obtained from CBA/H mice immunized against:	Lymphocytes removed from mice for MLC on days indicated after preimmunization:		
	15	30	60
	% specific ^{51}Cr release from DBA/2 targets		
CBA/H	18.6 (1)*	22.2 (1)	23.6 (1)
BRVR	3.8 (0.20)	13.0 (0.58)	19.2 (0.81)
BALB/c	26.6 (1.43)	37.3 (1.68)	44.5 (1.88)

Differences in specific ^{51}Cr release between the control and test immunized groups were significant within the range $P < 0.025$ to $P < 0.001$ estimated by analysis of variance tests.

Nonspecific kill estimated by isotope release from CBA/H in all experiments fell in the range of 0.02–1.65%.

* The percent ^{51}Cr release figures were transformed by expressing them relative to the syngeneic control which thus became unity.

TABLE III

Effect of Preimmunization on the Interaction of M Locus and H-2-Activated Clones in the Generation of Effectors Against M-Locus Targets

Cells for in vitro sensitization taken from CBA/H mice immunized against:	Lymphocytes taken from mice for in vitro sensitization with (CBA/H × DBA/2)F_1 cells on days indicated after preimmunization:			
	3	6	15	19
	% specific ^{51}Cr release from BRVR targets			
CBA/H	1.83	1.72	2.04	2.16
BRVR	0.22	0.88	1.12	3.55
BALB/c	0.95	1.54	2.23	1.06

All ^{51}Cr release data do not represent significant kill as determined by analysis of variance tests.

‡ PHA transformed; similar results were found with LPS-transformed targets.

finding that has been confirmed and extensively studied by Jacobsson et al. and Lilliehöök et al. (13, 15, 16); not only did they find that there was a suppressed MLR, but also a weak GVHR (17). It is not surprising, therefore, that preimmunization against *M* locus did not produce a consistent heightened reaction in a time-course study as was noted in the case of *H-2* preimmunization.

The suppression of response of H-2 antigens in *M* locus pre'mmunized mice cannot satisfactorily be explained by a pre-emption process of totipotential clones, as we suspect that different clones are activated by *H-2*- and *M*-locus LADs. Nevertheless, we have somehow to explain how the immunization by one genetic determinant suppresses the response to another. This could either be through the release of a factor (18) that suppresses the activation or through cell contact between a suppressor clone and the potentially activated clone causing negative co-operation or pre-emption by immune distraction, so instead of co-operating with helper T cells to produce effectors, the prekiller cell interacts with suppressor cells which inactivate it and prevent it from generating cytotoxic effectors.

In the time-course study of the effect of preimmunization an interesting resemblance was noted in the pattern of anti-*H-2* cytotoxicity produced by *H-2* complex LADs and *M* locus—there was a relative decline in the efficiency of amplification by *H-2* at the same time as the maximum suppressive action of the *M*-locus preimmunized lymphoid population. Moreover, periods of amplification produced by both types of immunization coincided. These observations imply that the immune response to both *M* locus and H-2 antigens is the integration of two opposing components, i.e. amplification and suppression, the latter component being predominant in the *M*-locus-immunized mice. The possibility also arises that *M* locus and *H-2* LADs are heterogeneous; one or more H-2 LADs may act as helpers for heightened reactivity, while others may be suppressive. Similarly, genes associated with the *M* locus may code for determinants predominantly suppressive in function. Experiments are in progress to examine these possibilities.

Summary

The effect of preimmunization of CBA/H mice with *M* locus and *H-2* incompatible lymphocytes was examined on the in vitro generation of cytotoxic cells to both types of targets. Preimmunization with *M* locus did not help to generate cytotoxic cells to *M*-locus targets; however, the anti-*H-2* cytotoxicity of *M*-locus-preimmunized lymphocytes was suppressed between the 6th and 19th day after preimmunization. In contrast, preimmunization with *H-2* incompatibility produced an amplified lytic anti-*H-2* response. The possible mechanisms are discussed.

We would like to thank the Cancer Research Campaign for financial support.

Received for publication 12 November 1975.

References

1. Festenstein, H., K. Abbasi, and P. Demant. 1974. The genetic basis of the generation of effector capacity for cell-mediated lympholysis in mice. *J. Immunogenetics.* 1:47.
2. Abbasi, K., and H. Festenstein. 1973. Antigenic strength investigated by cell-mediated lympholysis in mice. *Eur. J. Immunol.* 3:430.
3. Alter, B. J., and F. H. Bach. 1974. Role of H-2 lymphocyte defined and serologically defined components in the generation of cytotoxic lymphocytes. *J. Exp. Med.* 140:1410.
4. Forman, J., and J. Klein. 1975. Analysis of H-2 mutants: evidence for multiple CML target specificities controlled by the $H\text{-}2^{k,b}$ gene. *Immunogenetics.* 1:469.
5. Bach, F. H, M. B. Widmer, M. L. Bach, and J. Klein. 1972. Serologically defined and lymphocyte defined components of the major histocompatibility complex in the mouse. *J. Exp. Med.* 136:1430.
6. Festenstein, H. 1973. Immunogenetic and biological aspects of *in vitro* lymphocyte allotransformation (MLR) in the mouse. *Transplant. Rev.* 15:62.
7. Festenstein, H. 1974. Pertinent features of M locus determinants including revised nomenclature and strain distribution. *Transplantation (Baltimore).* 18:555.
8. Shreffler, D. C., and C. S. David. 1975. The H-2 major histocompatibility complex and the immune response. Genetic variation, function, and organization. *Adv. Immunol.* 20:125.

9. Schendel, D., B. Alter, and F. H. Bach. 1973. The involvement of LD- and SD-region differences in MLC and CML: a three cell experiment. *Transplant Proc.* 5:1651.
10. Röllinghoff, M., and H. Wagner. 1975. Effect of M locus dependent T-cell proliferation upon the induction of anti H-2 cytotoxic T effector cells. *J. Immunogenetics.* 2:301.
11. Adler, W. H., T. Takiguchi, B. Marsh, and R. J. Smith. 1970. Cellular recognition by mouse lymphocytes *in vitro*. II. Specific stimulation by histocompatibility antigens in mixed cell cultures. *J. Immunol.* **105**:984.
12. Virolainen, M., P. Hayry, and V. Defendi. 1969. Effect of presensitization on the mixed lymphocyte reaction of rat spleen cultures. *Transplantation (Baltimore).* 8:179.
13. Jacobsson, H., B. Lilliehöök, and H. Blomgren. 1975. Disappearance of specifically MLC responsive lymphocytes in CBA mice injected with cells from the H-2 compatible, M-antigen incompatible strain C3H. *Scand. J. Immunol.* 4:181.
14. Matossian-Rogers, A., P. Demant, and H. Festenstein. 1975. Further studies on the genetic basis of the generation of CML in mice. *J. Immunogenetics.* 2:291.
15. Lilliehöök, B., H. Jacobsson, and H. Blomgren. 1975. Specifically decreased MLC response of lymphocytes from CBA mice injected with cells from the H-2 incompatible, M-antigen-incompatible strain C3H. Lack of such effect after injection of H-2 disparate C3H-hybrid cells. *Scand. J. Immunol.* 4:209.
16. Jacobsson, H., B. Lilliehöök, and H. Blomgren. 1975. Capacity of C3H × CBA spleen cells to specifically depress the MLC—response of CBA mice. Evidence for an active role of the injected cells. In press.
17. Lilliehöök, B., and H. Blomgren. 1974. Weak graft-versus-host response of CBA lymphocytes against the H-2 identical strain C3H. *Scand. J. Immunol.* 3:637.
18. Zambala, M., and G. L. Asherson. 1975. *In vitro* absorption and molecular weight of specific T-cell suppressor factor. *Nature (Lond.).* 253:72.