Brief Definitive Report

MIXED LYMPHOCYTE REACTIVITY AND CELL-MEDIATED LYMPHOLYSIS TO TRINITROPHENYL-MODIFIED AUTOLOGOUS LYMPHOCYTES IN C57BL/10 CONGENIC AND B10.A RECOMBINANT MOUSE STRAINS

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Cell-mediated lympholysis (CML) to trinitrophenyl (TNP)-modified autologous splenic lymphocytes has been recently reported in the mouse (1). Both the sensitization and effector phases of this phenomenon were shown to be T-cell mediated. Effector cell specificity studies indicated that modification of the target cells is a necessary but insufficient requirement for cytolysis, and suggested that altered cell surface components controlled by genes mapping in the mouse major histocompatibility *H-2* complex (MHC) are important in the specificity of the cytotoxic reaction (1). In allogeneic models the generation of cytotoxic effector cells has been shown to be preceded or accompanied by immunogen-induced proliferation of responding lymphocytes, i.e. a mixed lymphocyte reaction (MLR) (2–5), although the generation of effectors may not necessarily always be the consequence of extensive cell proliferation (5). If the induction of cytotoxic effector lymphocytes by modified syngeneic spleen cells is characteristic of sensitization with cellular alloantigens, one would expect to find that sensitization with TNP-modified autologous cells would also induce thymidine incorporation by the responding cells in the culture. The present report demonstrates that both stimulation of thymidine incorporation and generation of cytotoxic effector cells are part of the in vitro response to TNP-modified autologous lymphocytes. However, the MLR to TNP-modified autologous cells consistently appeared to be less pronounced when compared with an allogeneic MLR, whereas the cytotoxic activity of the effector cells generated by sensitization against TNP-modified autologous cells was frequently as high as that detected against *H-2* alloantigens. These two components of reactivity to "modified self" are verified in several C57BL/10 congenic and B10.A recombinant mouse strains.

Materials and Methods

Mice. The C57BL/10 and B10 congenic resistant mouse strains used in this study were males, 6–8 wk of age, purchased from Jackson Laboratories, Bar Harbor, Maine. The B10.A recombinant mice used were raised in our mouse colony from breeding stock obtained from Dr. Donald C. Shreffler, Department of Human Genetics, University of Michigan, Ann Arbor, Mich.

Cell Cultures. Cell suspensions were prepared from the spleens of normal, unimmunized mice

and a portion of the suspension was reacted with trinitrobenzene sulfonate (Pierce Chemical Co., Rockford, Ill.) for 10 min at 37°C as described elsewhere (1). The TNP-modified and unmodified cell preparations were resuspended in RPMI-1640 media containing 10% fetal bovine serum, HEPES buffer (0.01 M), glutamine (0.03%), 2-mercaptoethanol (5×10^{-5} M final), and penicillin and streptomycin. Stimulating cells were exposed to 2,000 rads after TNP modification. For the mixed lymphocyte cultures, 5×10^5 responding spleen cells were incubated at 37°C in 7% CO_2 in 92-well Falcon plastic flat bottom microtiter plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) for 48 or 72 h, with 5×10^5, 7.5×10^5, or 1×10^6 irradiated, modified stimulating cells. The cultures were then incubated for an additional 24 h in the presence of 0.5 μCi [*methyl*-^{3}H]thymidine (New England Nuclear, Boston, Mass.) and counted in a β-scintillating spectrometer. The aliquots of 7×10^6 responding and 3×10^6 stimulating cells used for the generation of cytotoxic effector cells were prepared in the same culture media and incubated for 5 days in Linbro culture plates (Linbro Chemical Co., New Haven, Conn.) at 37°C in 7% CO_2. Effector cells were harvested from the cultures and assayed on ^{51}Cr-labeled, TNP-modified syngeneic spleen target cells as described (1). The method employed for calculation of the percent specific cytotoxicity has been outlined elsewhere (1).

Results and Discussion

The results summarized in upper portion of Table I are from experiments in which the MLR and CML were performed independently. The data shown in the lower part of the table represent the results of experiments in which the MLR and CML were performed on the same suspension of responding and stimulating cells. For MLR, the counts per minute with standard errors are shown for both the experimental (unmodified responders plus TNP-modified syngeneic or allogeneic stimulators) and control (unmodified responders plus unmodified syngeneic stimulators) groups as well as for the experimental:control ratios. The percentages of specific cytotoxicity with standard errors are given for the CML.

The MLR stimulation ratios obtained for TNP-modified autologous stimulator cells ranged from 1.62 to 3.04 among the various strains and experiments. These differences were all significant at $P < 0.01$. Stimulation of responding lymphocytes from the same pool with *H-2*-different allogeneic cells resulted in stimulation indices ranging from 2.02 to 9.08. In all groups of responding cells in which comparisons were made, allogeneic stimulation gave higher MLR than did stimulation with TNP-modified autologous spleen cells.

To determine whether the MLR stimulation indices obtained in the TNP-modified autologous cultures could be increased, the proportion of unmodified and modified irradiated and unirradiated spleen cells was varied. The results using normal and TNP-modified B10.BR spleen cells are summarized in Table II. An equivalent amount of stimulation was detected (experimental:control ratio of 2.49 vs. 2.32), when equal amounts of unmodified and TNP-modified spleen cells were cultured together, irrespective of which cell population was irradiated (upper part of Table II). When all cells in the mixture were TNP modified, half of them irradiated, the stimulation index increased to 4.21. These results suggest that the extent of [^{3}H]thymidine incorporation is directly proportional to the amount of TNP-modified cells in the culture mixture. In order to verify this, unequal numbers of unmodified and modified spleen cells were cultured together (lower part of Table II). Mixtures of 5×10^5 normal spleen cells with 2.5×10^5 modified, irradiated cells gave a stimulation index of 1.94. Incubation of the 5×10^5 modified, unirradiated spleen cells in the presence of 2.5×10^5 unmodified, irradiated spleen cells increased the stimulation index to 2.62. When all the cells

TABLE I

*Mixed Lymphocyte Reactivity and Cell-Mediated Cytotoxicity to TNP-Modified Autologous and Allogeneic Lymphocytes in C57BL/10 Congenic and B10.A Recombinant Mouse Strains**

Responding cells	Stimulating and target cells	MLR			Specific§lysis ± SE of cytotoxic assay
		Experimental	Control	Experimental‡: control	
		cpm ± SE	*cpm ± SE*		%
B10	B10-TNP	13,380 ± 902	4,407 ± 363	3.04	26.3 ± 5.3
	B10.D2	35,790 ± 1,517	4,959 ± 326	7.22	46.3 ± 3.5
B10.D2	B10.D2-TNP	7,186 ± 289	3,179 ± 176	2.26	37.2 ± 2.7
	B10.BR	17,200 ± 1,188	3,179 ± 176	5.41	41.8 ± 2.6
B10.BR	B10.BR-TNP	7,873 ± 717	4,578 ± 147	1.72	30.0 ± 2.5
	B10	9,258 ± 2,007	4,578 ± 147	2.02	25.6 ± 1.9
B10.A	B10.A-TNP	8,886 ± 617	5,485 ± 109	1.62	26.8 ± 3.0
	B10	30,250 ± 2,292	5,485 ± 109	5.52	26.4 ± 4.1
B10.A(2R)	2R-TNP	14,130 ± 1,119	4,839 ± 374	2.92	36.5 ± 4.0
	B10	37,650 ± 1,692	4,839 ± 374	7.78	Not tested
B10.A(4R)	4R-TNP	9,200 ± 937	3,278 ± 288	2.81	38.8 ± 5.3
	B10.D2	29,800 ± 1,722	3,278 ± 288	9.08	24.5 ± 3.0
B10.A(5R)	5R-TNP	7,960 ± 890	4,320 ± 78	1.84	33.0 ± 3.3
	B10.BR	12,700 ± 310	4,320 ± 78	2.94	56.7 ± 7.8
B10	B10-TNP	9,144 ± 518	5,675 ± 265	1.61	15.9 ± 3.0
	B10.D2	44,476 ± 1,939	5,675 ± 265	7.83	23.3 ± 1.7
B10.BR	B10.BR-TNP	15,509 ± 683	9,117 ± 485	1.70	37.1 ± 3.0
	B10	24,276 ± 1,809	9,117 ± 485	2.66	40.8 ± 1.8
B10.A	B10.A-TNP	11,565 ± 234	6,184 ± 234	1.87	31.0 ± 4.3
	B10	16,167 ± 194	6,184 ± 234	2.61	51.3 ± 3.9
B10.A(2R)	2R-TNP	12,668 ± 719	6,489 ± 1,358	1.95	31.2 ± 3.6
	B10	23,649 ± 901	6,489 ± 1,358	3.64	35.5 ± 2.2
B10.A(5R)	5R-TNP	6,485 ± 307	3,931 ± 165	1.65	13.9 ± 1.5
	B10.BR	22,124 ± 1,779	3,931 ± 165	5.63	40.2 ± 2.3

* Cell mixtures cultured for 48 h without [^{3}H]thymidine followed by an additional 24 h with [^{3}H]thymidine.

‡ $P < 0.01$ in all cases.

§ Spontaneous lysis of spleen TNP-target cells in the presence of unsensitized cultured cells was 21–30%. Effector:target cell ratio, 20:1.

‖ The data shown in the upper portion of the table are from experiments in which MLR and CML were tested independently. The data from the lower part of the table represent experiments in which MLR and CML were tested on portions of the same responding cell suspension. In all cases the TNP autologous and allogeneic MLR and/or CML were tested on aliquots from the same responding cell pool.

in the cultures were TNP modified (of which 5×10^5 were unirradiated and 2.5×10^5 were irradiated), the experimental:control ratio was increased to 4.51. These latter results confirm the data shown in the upper part of Table II, indicating that the amount of stimulation in the range tested is dependent on the number of cells in the culture which have been modified with TNP. These observations suggest that the extent of [^{3}H]thymidine incorporation is not dependent on the presence of two separate populations of cells which are distinguishable as a

TABLE II

*In Vitro [^3H]Thymidine Incorporation by Mixtures of Unmodified and TNP-Modified B10.BR Spleen Cells**

Cells in mixture ($\times 10^6$)		Counts per minute ± SE	Experimental‡: control
Unirradiated	2,000 R Irradiated		
5.0 B10.BR	5.0 B10.BR	11,720 ± 601	—
5.0 B10.BR	5.0 B10.BR-TNP	29,220 ± 1,147	2.49
5.0 B10.BR-TNP	5.0 B10.BR	27,180 ± 1,488	2.32
5.0 B10.BR-TNP	5.0 B10.BR-TNP	49,350 ± 3,456	4.21
0	5.0 B10.BR	230 ± 34	—
0	5.0 B10.BR-TNP	168 ± 6	—
5.0 B10.BR	2.5 B10.BR	14,770 ± 363	—
5.0 B10.BR	2.5 B10.BR-TNP	28,680 ± 833	1.94
5.0 B10.BR-TNP	2.5 B10.BR	38,760 ± 675	2.62
5.0 B10.BR-TNP	2.5 B10.BR-TNP	66,570 ± 1,502	4.51

* Cell mixtures cultured for 72 h without [^{3}H]thymidine followed by an additional 24 h with [^{3}H]thymidine.
‡ $P < 0.01$ in all cases.

population of unmodified, unirradiated responding lymphocytes and as a second population of TNP-modified irradiated stimulating cells. In this respect the system may be autoimmune-like. It has not been excluded, however, that the [^{3}H]thymidine incorporation in the TNP autologous model involves hapten-specific stimulation rather than reactivity against modified self as in the case of CML (1). It remains to be established whether these modified responding and stimulating cells can be distinguished from each other by T- and B-cell markers (6, 7).

T-cell-mediated cytotoxicity was also obtained for all mouse strains tested in which normal splenic lymphocytes were stimulated with TNP-modified autologous spleen cells. In contrast to the MLR comparisons, the levels of cytotoxicity generated against modified autologous cells was frequently equal to and sometimes higher than those detected against unmodified, allogeneic cells. It has been previously demonstrated that these effector cells are not exclusively hapten specific, but appear to recognize modified cell surface components controlled by genes mapping within the murine MHC (1). The finding that all C57BL/10 and B10.A recombinant lines tested can be altered by TNP to generate autoreactivity to modified self provides the opportunity for mapping the specificity of the effector cells within the *H-2* complex. In a preliminary study (8) the response of C57BL/10 spleen cells did not consistently generate effector cells to TNP-modified syngeneic spleen cells. More recently, however, (as shown here) the C57BL/10 strain has been as responsive as other strains. The genes controlling these TNP-modified self-antigens have recently been localized to the *K* and *D* serological regions of *H-2*.[1]

From the results obtained in this study it appears that some degree of cell proliferation may be required for or associated with generation of cytotoxic effector cells. The in vitro stimulation of normal splenic lymphocytes with TNP-

[1] Shearer, G. M., T. G. Rehn, and C. A. Gabarino. 1975. Cell-mediated lympholysis to trinitrophenyl-modified autologous lymphocytes: effector cell specificity to modified cell surface components controlled by the *H-2K* and *H-2D* serological regions of the murine major histocompatibility complex. *J. Exp. Med.* In press.

modified autologous spleen cells leads to cell proliferation as well as to the generation of cytotoxic effector cells. However, these results indicate that extensive cell proliferation (a strong MLR) is not necessary in the modified autologous model in order to generate effector cell cytotoxic potential equivalent to that detected with *H-2* allogeneic cell combinations. Isolated examples involving weak MLR and strong CML have been reported using an allogeneic cell combination, although they may be exceptional (5).

This system may be useful for further elucidation of the lymphocyte-defined (LD) and serologically-detectable (SD) MHC regions of both mice and men, since the method is being adapted for studying immune reactions of cultured human leukocytes. In mouse strain combinations for which only weak cellular immunity can be generated due to presumed absence of strong LD antigenic differences between responder and stimulator cells in the presence of SD differences (3, 9), it may be possible to "create" new LD differences by modification of stimulator cell surfaces, and thereby generate effector cells capable of lysing unmodified targets. Such a model might have some practical value, if the weak cell-mediated immunity observed to certain autologous tumor-specific antigens were attributable to the absence of adequate LD determinants necessary for initiation of the response.

The authors are grateful to Carol A. Garbarino for excellent technical assistance and to Marilyn K. Schoenfelder for preparation of the manuscript.

Received for publication 20 January 1975.

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