Brief Definitive Report

CELL-MEDIATED LYMPHOLYSIS TO *H-2*-MATCHED TARGET CELLS MODIFIED WITH A SERIES OF NITROPHENYL COMPOUNDS

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At least four different assays have been used to investigate the fine specificity of lymphocytes considered to possess T-cell characteristics. These methods include delayed hypersensitivity reactions in guinea pigs (1–4), in vitro proliferation of peritoneal lymphocytes in guinea pigs (3, 4) and mice (5), macrophage migration inhibition by lymphocytes (4), and the generation of cytotoxic T lymphocytes to viral-infected (6–9) or chemically modified (10, 11) autologous cells. In the latter system, two basic models for the cytotoxic T-cell receptor have been proposed (12, 13): one involving a single receptor by which the lymphocyte recognizes the infectious or modifying agent and self-*H-2* products as a single antigenic unit; and the other, in which the T lymphocyte expresses two distinct receptors, one for "hapten" and the second for autologous *H-2* products. By challenging T lymphocytes from guinea pigs immunized with hapten coupled to mycobacteria with a series of related haptens and carriers, Janeway and coworkers found that T cells recognized neither hapten nor carrier alone, but specificities involving both moieties (3, 4). An earlier report demonstrated that there was no detectable cross-reactivity within the same mouse strain between cytotoxic lymphocytes generated by sensitization with autologous cells modified with either trinitrobenzene sulfonate (TNBS) or *N*-(3-nitro-4-hydroxy-5-iodophenylacetyl)-β-alanylglycylglycyl (NIP-AGG) acyl azide (11). The present study further defines the fine specificity of cell-mediated lympholysis (CML) reactions against chemically modified autologous cells by comparing the specificity of effector cells generated by sensitization with autologous lymphocytes from C57BL/10 mice modified with the acyl azides of NIP-AGG, NP-AGG, NIP, TNP-AGG, and with TNBS (see Materials and Methods for explanation of abbreviations).

Materials and Methods

Mice. All mice used in these experiments were 7- to 9-wk-old C57BL/10 males obtained from The Jackson Laboratory, Bar Harbor, Maine.

Tumor Cells. The only tumor used in these experiments was L5MF-22 (*H-2*^{b}), a radiation-induced lymphoma which arose in a B10.129(5M) female mouse (obtained from Dr. Gustavo Cudkowicz, Department of Pathology, State University of New York, Buffalo, N. Y.) and was passed at 7- to 9-day intervals as ascitic fluid in C57BL/6 mice.

Chemical Modifications of Spleen and Tumor Cells. TNP modification of cells was performed

as previously described (10) using a 10 mM TNBS solution (Pierce Chemical Co., Rockford, Ill.) in phosphate-buffered saline, pH 7.4.

All other compounds were covalently bound to cells using haptens in the form of an acyl azide as previously described for NIP-AGG (11, 14, 15). These compounds included: *N*-(3-nitro-4-hydroxy-5-iodophenylacetyl)-β-alanylglycylglycyl (NIP-AGG) azide; *N*-(3-nitro-4-hydroxyphenylacetyl)-β-alanylglycylglycyl (NP-AGG) azide; *N*-(3-nitro-4-hydroxy-5-iodophenylacetyl) (NIP) azide; and *N*-(2,4,6-trinitrophenyl)-β-alanyglycylglycyl (TNP-AGG) azide.

In Vitro Cell Culture and Assay for Cytotoxic Cells. Splenic lymphocytes were sensitized in vitro with chemically modified spleen cells in supplemented RPMI 1640 media as previously described (11). After a 5 day incubation the cells were harvested and their cytotoxic activity measured as previously described (10). Nonspecific lysis of modified target cells was determined from the amount of ^{51}Cr released from the appropriate ^{51}Cr-labeled target cells, incubated for 4 h with effector cells which had been incubated 5 days with no stimulators, divided by the maximum ^{51}Cr released by targets that had been frozen, and thawed repeatedly.

Results

Spleen cells from C57BL/10 mice were sensitized in vitro with autologous spleen cells modified with TNBS or with the acyl azide of TNP-AGG. The effector cells generated 5 days later were assayed on ^{51}Cr-labeled L5MF-22 (*H-2*^{b} lymphoma target cells modified with either TNBS or TNP-AGG acyl azide. The results of five independent experiments (Table I) indicate that effector cells sensitized with autologous TNP-modified cells which consistently lysed *H-2*-matched, TNP-modified targets never lysed *H-2*-matched, TNP-AGG-modified target cells. It was demonstrated in four of the five experiments that TNP-AGG-modified targets were capable of being lysed, since effectors generated by sensitization with TNP-AGG-modified autologous cells lysed *H-2*-matched, TNP-AGG-modified target cells. A variable degree of lysis was obtained from experiment to experiment when these same effectors were tested on *H-2*-matched, TNP-modified targets. This degree of lysis ranged from 75 (exp. 5-387) to 6% (experiment 5-439) of the lysis obtained on TNP-AGG-modified targets. It is noteworthy that sensitization against autologous cells modified with TNP-AGG acyl azide was always weaker than with cells modified with TNBS.

In another series of experiments the specificity of C57BL/10 cytotoxic effector cells was investigated as a function of sensitization with autologous spleen cells modified with the acyl azides of NIP-AGG, NP-AGG, or NIP. The results of four independent experiments are summarized in Table II. In all cases effector cells sensitized with autologous NIP-AGG-modified spleen cells lysed *H-2*-matched, NIP-AGG-modified tumor targets to a greater extent than tumor targets modified with the closely related acyl azides of NP-AGG or NIP. Tumor target cells modified with TNP-AGG were not lysed to any appreciable extent by these effectors.

NP-AGG-modified autologous cells were only marginally immunogenic as sensitizing cells since weak responses were detected in only two out of four experiments. In experiments 5-451 and 5-413, in which sensitization to NP-AGG-modified cells was obtained, variable cross-reactivity was observed with NIP-AGG-modified targets, and no cross-reactivity was obtained with NIP-modified targets.

Sensitization of C57BL/10 spleen cells with NIP-modified autologous cells generated effectors which lysed NIP-modified targets in all four experiments.

TABLE I

In Vitro Induction of Cytotoxicity of C57BL/10 Spleen Cells by TNP-Modified or TNP-AGG-Modified Spleen Cells to TNP-Modified and TNP-AGG-Modified L5MF-22 Tumor Target Cells

Stimulating C57BL/10 spleen cells modified with:	L5MF-22 target cells modified with:	% Specific lysis + SE in exp. no.:				
		5-387*	5-407*	5-412*	5-419*	5-439*
TNP	TNP	45.2 ± 1.7	30.3 ± 2.2	45.6 ± 2.1	27.6 ± 2.0	39.6 ± 2.9
TNP	TNP-AGG	6.4 ± 2.3	1.6 ± 2.5	NT‡	(−4.0 ± 3.2)	2.0 ± 2.9
TNP-AGG	TNP-AGG	11.5 ± 2.7	25.6 ± 4.6	22.2 ± 2.3	NT	13.0 ± 2.3
TNP-AGG	TNP	8.6 ± 1.7	NT	11.9 ± 2.0	NT	0.8 ± 0.4

* Experiment number, effector:target ratio: 5-387, 80:1; 5-407, 20:1; 5-412, 40:1; 5-419, 40:1; and 5-439, 160:1.
‡ NT, not tested.

TABLE II

In Vitro Induction of Cytotoxicity of C57BL/10 Spleen Cells by NIP-AGG-, NP-AGG-, or NIP-Modified Spleen Cells to NIP-AGG-, NP-AGG-, or NIP-Modified L5MF-22 Tumor Target Cells

Stimulating C57BL/10 spleen cells modified with:	L5MF-22 target cells modified with:	% Specific lysis ± SE in exp. no.:			
		5-413*	5-419*	5-439*	5-451*
NIP-AGG	NIP-AGG	54.1 ± 2.1	37.0 ± 1.6	24.8 ± 3.3	43.6 ± 1.3
NIP-AGG	NP-AGG	22.3 ± 2.5	17.5 ± 2.1	2.0 ± 1.2	13.9 ± 1.3
NIP-AGG	NIP	4.1 ± 2.6	10.7 ± 2.9	6.3 ± 3.4	10.1 ± 2.3
NIP-AGG	TNP-AGG	NT‡	1.6 ± 3.6	2.9 ± 1.4	5.3 ± 2.1
NP-AGG	NIP-AGG	6.1 ± 1.3	(−13.8 ± 1.9)	(−7.6 ± 0.9)	11.1 ± 1.7
NP-AGG	NP-AGG	11.1 ± 2.4	(−11.3 ± 1.8)	(−4.7 ± 1.6)	12.6 ± 1.2
NP-AGG	NIP	0.0 ± 4.1	(−1.9 ± 3.5)	(−5.2 ± 1.6)	0.7 ± 1.8
NIP	NIP-AGG	11.9 ± 2.0	(−6.0 ± 1.9)	2.7 ± 1.3	8.1 ± 1.1
NIP	NP-AGG	3.6 ± 2.0	(−1.4 ± 1.4)	0.3 ± 1.1	5.6 ± 0.5
NIP	NIP	10.1 ± 1.2	18.7 ± 2.5	28.8 ± 3.7	20.2 ± 1.9

* Experiment number, effector target ratio: 5-413, 80:1; 5-419, 160:1; 5-439, 80:1; and 5-451, 160:1.
‡ NT, not tested.

No lysis by these effectors was detected on NP-AGG-modified targets in any of the experiments. These effectors clearly lysed NIP-AGG-modified targets only in experiment 5-413.

In order to further characterize the specificity of the cytotoxic lymphocytes, cold target inhibition experiments were performed in which nonradioactive cells modified with these reagents were used to block the effector phase of the CML (Table III). Effector lymphocytes were generated by sensitization of C57BL/10 spleen cells with either TNP-modified (section A) or NIP-AGG-modified (section B) autologous spleen cells, and assayed on ^{51}Cr-labeled, *H-2*-matched, L5MF-22 target cells modified with TNBS or NIP-AGG acyl azide, respectively. The results shown in section A demonstrate that only TNP-modified spleen cells were effective inhibitors of the cytotoxic reaction. In contrast, TNP-AGG-modified spleen cells were no more effective in blocking cytolysis than were unmodified C57BL/10 spleen cells. The lysis by effector cells generated by sensitization of C57BL/10 splenic lymphocytes with NIP-AGG-modified autologous-stimulating cells was inhibited by NIP-AGG-modified spleen cells, but not by unmodified, NP-AGG-modified, nor NIP-modified spleen cells (section B). It is important to note that NP-AGG-modified cells were effective targets in this same

TABLE III

Effect of Preincubation of Chemically Modified, Nonradioactive Spleen Cells With C57BL/10 Effector Cells on the Lysis of Chemically Modified L5MF-22 Tumor Target Cells*

Chemical modification of C57BL/10 stimulator spleen cells and L5MF-22 target cells	Blocker:target cell ratio	% Specific lysis ± SE in presence of nonradioactive C57BL/10 spleen cells modified with:			
		Not modified	TNP	TNP-AGG	
A					
TNP	40:1	34.6 ± 1.9	31.2 ± 2.4	37.4 ± 5.4	
	80:1	27.0 ± 2.8	16.9 ± 2.7	33.1 ± 1.9	
	160:1	21.9 ± 2.3	12.5 ± 1.4	27.6 ± 1.3	
		Not modified	NIP-AGG	NP-AGG	NIP
B					
NIP-AGG	20:1	24.3 ± 2.1	15.1 ± 2.3	26.1 ± 0.2	26.0 ± 0.6
	40:1	15.7 ± 2.7	4.8 ± 1.3	23.8 ± 1.4	24.0 ± 2.0
	80:1	16.6 ± 1.6	9.3 ± 1.4	24.5 ± 2.1	23.4 ± 1.5
	160:1	15.8 ± 1.2	8.5 ± 1.6	22.2 ± 1.5	20.1 ± 1.8

Effector:Target Ratio of section A, 20:1; section B, 40:1. Controls: section A, effector cell lysis of TNP-modified L5MF-22 targets: 45.6 ± 2.1%; section B, effector cell lysis of: (*a*) NIP-AGG-modified L5MF-22 targets, 27.6 ± 2.9%; (*b*) NP-AGG-modified L5MF-22 targets, 9.5 ± 2.3; (*c*) NIP modified L5MF-22 targets, (−0.3 ± 3.6).

* Inhibitor cells were preincubated for 30 min at 37°C with the effector cells before addition of ^{51}Cr-labeled target cells.

experiment, but they were not effective blockers for cytotoxic cells generated by sensitization with NIP-AGG-modified cells when assayed on NIP-AGG-modified targets (see footnote of Table III).

Discussion

The results presented in this report demonstrate that these cytotoxic lymphocytes recognize more than just the dominant terminal feature of the modifying agent or "hapten." In Table I, data are presented showing that cytotoxic lymphocytes generated by sensitization of splenic lymphocytes with TNP-modified autologous spleen cells lysed *H-2*-matched, TNP-modified target cells but not modified target cells in which the TNP group was separated from the cell surface by an alanylglycylglycyl tripeptide spacer. *H-2*-matched, TNP-AGG-modified nonradioactive targets did not inhibit the lysis of TNP-modified targets by TNP-self-sensitized effector cells (Table III). Such results are compatible with previous findings in the chemically modified CML model in which cytotoxic T lymphocytes were generated in vitro by sensitization with chemically modified autologous cells (10, 11, 13). These findings include the observations that: (*a*) *H-2* homology is required between stimulator and target cells (10, 11, 13); (*b*) TNP-modified and NIP-AGG-modified stimulating cells generate clones of effectors which can distinguish between these two modifying agents (11); (*c*) cold target inhibition can occur only when the competing, nonradioactive target cells are both *H-2*-matched and modified with the same agent as the stimulating cells (11, 13); and (*d*) inhibition of the lysis of modified target cells is obtained by anti-H-2 sera directed against the modified targets (16). Furthermore, the observations presented in this report are compatible with the results of Janeway and co-workers using related nitrophenyl compounds (3, 4) in which specificity was shown to include both the nitrophenyl groups and part of the protein carrier.

It was found in the current study, however, that effector cells generated by sensitization with TNP-AGG-modified stimulating cells could lyse TNP-modified targets in some but not all experiments (see lower part of Table I). These findings, which appear to contradict the observation that TNP-AGG-modified targets were never lysed by effector cells generated by sensitization with TNP-modified autologous stimulating cells, might be accounted for if sensitization with TNP-modified cells stimulates only clones of lymphocytes which recognize TNP and some portion of autologous *H-2* cell surface products. Sensitization with TNP-AGG-modified self would be expected to activate clones of lymphocytes recognizing TNP-AGG and some part of autologous *H-2* cell surface products. The cross-reactivity with TNP-modified cells could be accounted for by either the generation of a clone of effector cells which could lyse either TNP-AGG-self or TNP-self (cross-reactive clone). An alternative possibility is that sensitization with TNP-AGG-self could activate two groups of noncross-reacting clones: one which recognizes only TNP-AGG-self and a second which recognizes only TNP-self. These latter clones could be the same ones which are stimulated by TNP-self.

Effector cells generated by sensitization with NIP-AGG-modified cells consistently lysed NIP-AGG-modified targets to a greater extent than either NP-AGG- or NIP-modified target cells. The lower levels of lysis detected by either NP-AGG- or NIP-modified targets could also be accounted for by at least two mechanisms. First, a clone of effector cells could be generated by sensitization with NIP-AGG which could lyse all three targets (cross-reactive clones). Second, multiple, distinct, noncross-reacting clones could be activated by NIP-AGG-modified stimulating cells. The latter possibility implies that NIP-AGG-modified stimulating cells can stimulate clones of effector cells unable to lyse NIP-AGG-modified target cells. The blocking data shown in section B of Table III support the second possibility. Thus, the population of effector cells generated by sensitization with NIP-AGG-modified stimulating cells were capable of lysing NIP-AGG-modified target cells (27.6%) and to a lesser extent NP-AGG-modified target cells (9.5%) at an effector:target ratio of 40:1 (see footnote of Table III). The clones of effector cells which lyse NIP-AGG-modified target cells were blocked only by NIP-AGG-modified inhibitor cells. NP-AGG-modified cells did not inhibit this reaction even at a blocker:target ratio of 160:1. This suggests the presence of separate clones of effector cells, each of which distinguishes very specifically between NIP-AGG- and NP-AGG-modified target cells even though all clones resulted from stimulation by the same modifying agent. Thus, there can be a difference in discriminating ability or fine specificity toward the modifying group for the stimulating and lytic processes. Parallel differences in discriminating power have been frequently observed by Merchant and Inman (unpublished observations) in the case of B-cell activation leading to antibody production, and subsequent hapten-directed hemolytic lysis (in complement-dependent plaque assays). The inability of NIP-AGG-sensitized effector cells to lyse TNP-AGG-modified target cells demonstrates that AGG is not an exclusive immunodeterminant in this reaction. The inability of NP-AGG-modified cells to stimulate effector cells demonstrates the importance of the iodo group for immunogenicity in this system.

The data presented do not support the dual receptor model for T-cell recognition in its simplest form. The intimacy or dual receptor model has been proposed as a possible explanation for the *H-2* requirement for CML in the viral-infected (12) and chemically modified systems (13). The hypothesis states that the T-cell has two distinct receptors which must be accommodated for cytotoxicity to occur. One receptor is for the simple "hapten" (a hapten as defined in the antibody system would be TNP) and the other receptor would involve recognition of a self-*H-2* product on the target cell surface. If this model were valid, one would expect to see no example in which an effector cell would distinguish between a hapten coupled to a target cell surface and a hapten coupled to a target cell surface with a short flexible peptide spacer. For CML responses to TNP-modified autologous cells, this two-receptor model in its simplest form should involve the recognition of TNP hapten in one receptor and a self-*H-2* cell surface product in the other receptor. An effector cell sensitized to TNP-self would be expected to kill equally well TNP- or TNP-AGG-modified target cells, since both would fulfill the requirements of possessing the hapten TNP and self-*H-2* cell surface products. In order for the intimacy model to remain viable in the chemically modified CML system it is necessary to postulate that the so called hapten involves more than just TNP (which is a hapten for antibody production) and would have to include TNP plus one or more adjacent amino acids.

Summary

The specificity of C57BL/10 cytotoxic effector cells generated by in vitro sensitization with autologous spleen cells modified with a series of related nitrophenyl compounds was investigated. The failure of trinitrophenyl (TNP)-sensitized effector cells to lyse TNP-β-alanylglycylglycyl(AGG)-modified target cells is presented as evidence contradicting the intimacy or dual receptor model of T-cell recognition in its simplest form. Data are also shown indicating that sensitization with *N*-(3-nitro-4-hydroxy-5-iodophenylacetyl)-AGG-modified stimulating cells generates noncross-reacting clones of cytotoxic effector cells.

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