THE GENERATION OF KILLER CELLS TO TRINITROPHENYL-MODIFIED ALLOGENEIC TARGETS BY LYMPHOCYTE POPULATIONS NEGATIVELY SELECTED TO STRONG ALLOANTIGENS*

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Several studies have demonstrated the importance of genes of the major histocompatibility complex (MHC)[1] in the functional specificity of thymus-derived (T) lymphocytes. In mice, cytotoxic T lymphocytes (CTL) raised against minor histocompatibility antigens (1, 2), chemically modified target cells (3), tumor-associated antigens (4), or virally infected cells (5), display lytic activity against appropriate antigen-modified target cells only if these targets express determinants controlled by the MHC *K* and/or *D* regions of the original stimulating populations. Two models, "altered self" and "dual recognition" have been advanced to explain this control over T-cell function by MHC genes (5–7). The first suggests that CTL employ a single receptor to recognize neodeterminants created by the physical association of the antigen with self *K* and *D* region gene products on the target cell surface. The second holds that two receptors on CTL are involved, one specific for the modifying antigen, and a second one which recognizes self *K* and *D* gene products. A variation of this second model suggests that "like-like" interactions between MHC-coded "cell-interaction" determinants present on both killer and target cells are required for lytic expression of CTL. Despite considerable controversy and effort, definitive evidence favoring one or the other of these theories is not yet available.

In considering this question, it would be of interest to determine (*a*) if allogeneic MHC gene products exert a similar restrictive influence over induction and expression of CTL against appropriately modified allogeneic, MHC-incompatible targets, and (*b*) whether elimination of lymphocytes reactive to a particular MHC alloantigen affect the capacity to generate CTL activity against modified allogeneic target cells. The main problem with this approach is that much of the cytolytic activity generated against modified allogeneic targets can

* Supported by U. S. Public Health Service grants AI-10961 and CA-18522.

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[1] *Abbreviations used in this paper:* CTL, cytotoxic T lymphocytes; FCS, fetal calf serum; LN, lymph nodes; MHC, major histocompatibility complex; MLC, mixed lymphocyte cultures, MR, maximum release; SR, spontaneous release; TDL, thoracic duct lymphocytes; TNP, trinitrophenyl.

be expected to be directed towards target alloantigens per se. This problem can be avoided by using T cells that have been rendered unresponsive to the alloantigens in question. Failure of such "tolerant" T-cell populations to respond to modified allogeneic targets would indicate the importance of anti-MHC receptors in the recognition of antigenically altered allogeneic target cells, and thus would be strong presumptive evidence for a two receptor model.

Three groups have recently adopted this approach and have demonstrated that strain A lymphocytes tolerized to specific *H-2K* and *D* determinants of strain B in bone marrow chimeras are fully responsive to virally and chemically altered strain B target cells (8–10). At face value, these data argue against the two receptor model for the specificity of CTL. However, as Zinkernagel has suggested, a dual receptor model cannot be ruled out by these data since the chronic exposure of A cells to B-strain alloantigens in a chimeric environment may have permitted "adaptive differentiation" of these tolerant A cells in such a way that they are able to recognize strain B MHC antigens as "self" determinants (10); they might, for example, come to express strain B cell interaction determinants thus enabling like-like interactions with strain B target cells.

These considerations emphasize the necessity for rendering T cells unresponsive to alloantigens by a procedure which does not allow putative adaptive differentiation to occur. It would seem reasonable that this problem might be avoided with the use of an acute procedure for specific depletion of alloreactivity. The procedure adopted in this study is the "negative selection" system described by Ford and Atkins (11) in which parental strain mouse and rat T cells are depleted of reactivity to specific alloantigens by "filtration" from blood to lymph in irradiated F_1 hybrids. These filtered T cells were then used as the starting population to generate killer cells against trinitrophenyl (TNP)-modified allogeneic targets.

Materials and Methods

Animals. Young adult mice of the C57BL/6 (*H-2*^{b}) and CBA (*H-2*^{k}) strains and their F_1 hybrids were obtained from The Jackson Laboratory, Bar Harbor, Maine and from the Cumberland Laboratories, Clinton, Tenn. Rats of the DA (*Ag-B4*) and Fischer 344 (F; *Ag-B1*) strains and their F_1 hybrids were obtained from stocks maintained in our colonies.

Lymphocytes. Mouse and rat lymphocyte suspensions were obtained from lymph nodes (LN), spleens, and by thoracic duct cannulation. Suspensions were prepared in Hanks' balanced salt solution buffered to pH 7.0 with 0.01 M phosphate and supplemented with 2% (vol/vol) fetal calf serum.

Negative Selection. Lymphocyte populations specifically depleted of alloreactivity to a particular MHC haplotype were prepared according to procedures fully described elsewhere for rats (11, 12) and mice (13). Briefly, this involved acute "filtration" of parental strain (A) thoracic duct lymphocytes (TDL) (for rats) or LN suspensions (for mice) through irradiated (450 rads, rats; 900 rads, mice) A × B F_1 recipients. Lymph was then collected from the F_1 recipients over the period 8–30 h after injecting the parental cells; virtually all (98%) of collected cells at this time were shown previously (13) and in the present studies to be T cells of donor origin. The completeness of negative selection was determined with mixed lymphocyte cultures (MLC); negatively selected responder lymphocytes (A_{-B}) were tested with irradiated (2,000 rads) stimulator spleen cell populations of the original selecting MHC haplotype (B_x) and with third-party haplotypes (C_x, D_x, E_x . . .). In all instances, A_{-B} plus B_x cultures showed no proliferative activity over control levels while third-party responses (e.g. A_{-B} plus C_x) were normal. Previous work has shown that the unresponsiveness of negatively selected cells in MLC (12, 13) also applies to the capacity of these cells to produce killer cells (14), to cause graft-vs.-host reactions (11, 12), and to cause rejection of skin allografts (15).

Generation of Killer Cells

MOUSE CONDITIONS. These have been described in detail elsewhere (16). In brief, 10 million parental or F_1 LN cells or negatively selected TDL were stimulated with 8×10^6 X-irradiated (1,750 rads) TNP-modified syngeneic or allogeneic spleen cells. The cells were cultured in Falcon no. 3012 flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in 4.5 ml RPMI 1640 medium supplemented with fetal calf serum (FCS) [25% (vol/vol)], HEPES (12 mM), glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 2-mercaptoethanol (4×10^{-5} M; Eastman Kodak Co., Rochester, N. Y.).

RAT CONDITIONS. Responder populations (20 million) of parental TDL were stimulated with X-irradiated (2,000 rads) syngeneic and allogeneic LN cell populations (15 million) that were either normal or had been TNP modified. The cultures (2 ml) were conducted in the inner chamber of Marbrook vessels separated from an outer reservoir (30 ml) by dialysis membrane. The same media described above for mouse cultures were used except that media for the inner chamber were supplemented with 5% (vol/vol) fresh BN rat serum, while media of the outer chamber contained 5% FCS.

Both rat and mouse stimulator populations were coupled with TNP according to procedures described by Shearer (3). Cultures were maintained for 5 days (mouse) or for 6 days (rats) in a humid atmosphere of 7% CO_2 in air.

When rat cultures were terminated, dead cells and culture debris were removed by centrifugation (1,500 *g*, 15 min) on a discontinuous Ficoll-Isopaque gradient (12 parts 14% wt/vol Ficoll 400, Pharmacia Fine Chemicals, Inc., Piscataway, N. J., and 5 parts 32.8% wt/vol sodium metrizoate, Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.) according to procedures described by Davidson and Parish (17).

Killer Cell Assays. Target cells were LN lymphocytes that had been stimulated 3 days with concanavalin A (rat) or spleen cells stimulated for 2 days with lipopolysaccharide (mice). The cells were labeled with ^{51}Cr, then treated with TNP according to standard procedures.

Killer cell assays were conducted in V-bottom microtiter plates with 10^4 target cells at killer:target cell ratios from 1 to 40:1 (mice) and from 20 to 200:1 (rats). ^{51}Cr-release values were determined after 4 h of incubation (16, 18).

Results

Two experiments conducted with *H-2* incompatible mouse strains (Table I) and two experiments with *Ag-B* incompatible rat strains (Table II) gave similar results. These can be summarized as follows:

(Exp. 1). Cultures of normal (i.e., unselected) parental strain (A) lymphocytes stimulated with chemically modified syngeneic cells (A-TNP), as expected, developed CTL activity specific for modified syngeneic (A-TNP) targets. Similarly, lymphocytes from A × B F_1 donors cultured with A-TNP or B-TNP (done only in mice) developed CTL activity which was preferentially limited to modified target cells of the stimulating parental strain; in this situation, however, considerable degrees of cross-reactive killing were observed in one direction (F_1 cells stimulated with C57BL-TNP). Parental (A) cells cultured with modified allogeneic (B-TNP) stimulators developed lytic activity against both B and B-TNP target cells.

(Exp. 2) Parental strain (A) lymphocytes negatively selected (A_{-B}) to a particular MHC haplotype (B), as expected, failed to generate CTL activity against B or B-TNP targets when stimulated with either chemically modified strain A cells (Tables I and II) or unmodified (Table II) strain B cells.[2] In the case of unmodified stimulator cells, this also applied even when B × C F_1 stimulators

[2] In the second mouse experiment (Table I) the lysis of C57BL targets (12%) by CBA_{-C57} responder cells stimulated with C57BL-TNP seems to be of doubtful significance since comparable lysis (7%) of the same targets was also observed when F_1 lymphocytes were used as responders.

TABLE I

Generation of CTL Activity Against TNP-Modified Allogeneic Target Cells in Negatively Selected Mouse Lymphocyte Populations

Responder	Stimulator	Percent specific ^{51}Cr release*							
	Target	CBA		CBA-TNP		C57BL		C57BL-TNP	
	Exp.	I	II	I	II	I	II	I	II
	MR(cpm)	1,669	855	2,308	672	1.574	1,309	3,339	1,126
	SR(%)	29	50	26	32	32	28	30	25
CBA	CBA-TNP	5	0	60	57	3	−2	6	8
	C57BL-TNP	4	4	17	17	40	51	36	60
	F_1-TNP	4	1	45	54	36	40	26	53
(CBA × C57BL)F_1	CBA-TNP	6	0	43	57	0	−2	6	18
	C57BL-TNP	5	19	27	37	5	7	45	66
	F_1-TNP	6	13	46	53	0	0	21	32
CBA_{-C57BL}	CBA-TNP	2	6	46	58	−4	−2	0	16
	C57BL-TNP	0	3	21	43	3	12	26	62
	F_1-TNP	2	5	51	67	−5	−1	15	26

* CTL:target cell ratio 5:1.

TABLE II

Generation of CTL Activity Against TNP-Modified Allogeneic Target Cells in Negatively Selected Rat Lymphocyte Populations

Responder	Stimulator	Percent specific ^{51}Cr release*							
	Target	DA		DA-TNP		F		F-TNP	
	Exp.	I	II	I	II	I	II	I	II
	MR(cpm) .	1,449	1,146	1,339	1,102	1,602	786	1,664	701
	SR(%)	12	23	11	22	10	24	11	23
DA	DA-TNP	0	0	33	14	3	0	3	0
	F	0	0	0	1	27	30	26	33
	F-TNP	0	0	2	0	42	31	27	32
DA_{-F}	DA-TNP	0	−1	51	12	0	0	0	0
	F	0	0	0	0	0	−2	0	−1
	F-TNP	0	0	2	7	0	0	21	19
F	F-TNP	0	0	8	3	0	0	31	21
	DA	21	27	19	30	0	−1	0	0
	DA-TNP	25	29	22	29	0	0	2	0
F_{-DA}	F-TNP	0	0	7	13	3	–	25	40
	DA	0	−1	0	0	0	0	0	0
	DA-TNP	0	1	21	21	1	1	2	4

* CTL:target cell ratios 20–200:1; Cr-release values indicated represent the CTL:target cell ratio of 100:1 derived by linear interpolation of three point semilogarithmic plots.

were used (data not shown); thus, CBA_{-C57BL} responders cultured with C57BL × DBA/2 F_1 stimulators produced strong lysis against the third-party (DBA/2) targets and no activity against the specific (C57BL) targets.

Although A_{-B} lymphocytes cultured with B-TNP stimulators failed to lyse B targets, they did lyse B-TNP targets. In rats, this lysis was highly specific, the

lysis of modified syngeneic targets (A-TNP) being very low. However, in mice considerable cross-reactivity was observed. Here it should be noted that the cross-reactive lysis of CBA-TNP targets by $CBA_{\rightarrow C57BL}$ anti-C57BL-TNP cells was roughly comparable to that observed when CBA × C57BL F_1 cells were used as responders under similar conditions. Extensive cross-reactivity has been reported by others (19, 20).

Discussion

These experiments show that lymphocyte populations negatively selected to a particular MHC haplotype to the extent that they no longer react to that haplotype by any parameter yet studied can nevertheless generate killer cells against chemically modified allogeneic target cells expressing the selecting haplotype. These killer cells show the same degree of MHC-controlled restriction described for killer cells directed to modified syngeneic targets, i.e., they are effective against targets only if these are suitably modified and also bear the MHC haplotype of the original stimulating population.

The most direct implication of these data is that T cells having overt reactivity to a given MHC alloantigen do not play a significant role in MHC-controlled lysis of chemically modified target cells which express that alloantigen. While such a conclusion does not definitively distinguish between altered self and the dual recognition models for cytotoxic T-cell recognition, it does rule out two versions of the dual receptor model. The present studies involving acute specific depletion, and the studies of others using tolerant lymphocyte populations from chimeric mice (8–10), exclude a requirement to like-like interactions between killer and target cells as the basis of MHC restriction. In addition, they discount the notion that the response of T cells from chimeric mice to modified allogeneic targets is the outcome of an aberrant phenomenon involving adaptive differentiation of abnormal surface structures on the responding cell. Rather it appears that the potential for specific reactivity to modified allogeneic cells is present in the initial lymphocyte population.

At face value, the results of the present studies might be taken to support the altered self hypothesis with the added proviso that recognition of "altered" MHC determinants applies not only to self-determinants but also to alloantigens. While the results are certainly consistent with this view, one version of the dual recognition model cannot yet be discounted. The possibility remains that parental lymphocytes with low affinity receptors for alloantigens were not removed during the negative selection procedure (see also reference 7). Such cells, unable to be triggered and to express lytic activity to unmodified allogeneic targets, would resemble putative T-cell subpopulations with low affinity receptors for self-MHC gene products whose stimulation is postulated to require the presence of additional antigenic determinants.

It is of some interest that recent studies of Schmitt-Verhulst and her colleagues (A-M. Schmitt-Verhulst, personal communication) have produced contrasting results using BUDR and light to eliminate specific alloreactive cells in MLC. Cells negatively selected by this procedure failed to generate CTL activity also against chemically modified allogeneic target cells. The discrepancy between these results obtained with the different procedures for negative selection has yet to be resolved.

Summary

Negatively selected mouse and rat lymphocyte populations, specifically deprived of alloreactivity to a particular major histocompatibility complex (MHC) haplotype, are nevertheless fully capable of responding to trinitrophenyl (TNP)-modified allogeneic stimulator cells and developing cytotoxic T-lymphocyte activity to TNP-altered allogeneic target cells. As for syngeneic systems, lytic expression of those responder killer cells also requires MHC identity between the target and stimulator cell populations. Such a finding argues strongly against two variations of the dual recognition hypothesis: like-like interactions and adaptive differentiation. Instead, these data favor either the altered self model or a third variation of the dual receptor model, where one of the relevent receptors is specific for the modifying antigen and the second is a low affinity receptor unable to be triggered in the absence of a modifying antigen.

Received for publication 8 April 1977.

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