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Antigenic and Genetic Parameters in the Stimulation and in the Lytic Phases of Anti-Hapten + Self Cytotoxic T cells and Their Derived Clones: Role of the T helper cell

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Requirements for stimulation of cytotoxic T cells (CTL) and for their lytic recognition have been compared in T cell lines repeatedly stimulated with trinitrobenzene sulfonate-treated syngeneic murine spleen cells. Differences were observed between the requirements for cells to stimulate or to be lysed by the CTL, which included: (a) the expression of major histocompatibility complex (MHC = H-2) encoded allelic products, and (b) the hapten density. Propagation of the CTL within the line required I-A intra-H-2 homology between hapten-treated stimulating cells and the line cells, whereas the lytic interaction required H-2K region homology between hapten-treated target cells and CTL. The hapten density requirement was analyzed for a responder ($H\text{-}2^k$) and a non-responder ($H\text{-}2^b$) strain to low hapten density modified syngeneic cells. This property was found to be a characteristic of the lytic phase rather than of the stimulation of CTL. CTL clones could be derived by growing the line cells under conditions of limiting dilution in the presence of T cell growth factors. Such CTL clones were unable to be stimulated by their target antigens and were dependent on T cell growth factors for their propagation. These results are discussed in terms of the dependence of the development and growth of CTL on T helper cells.

Key words: anti-hapten + self CTL, T helper, CTL clones, (non)-responder strain

The generation of cytotoxic T cells (CTL) after in vitro sensitization with allogeneic cells or with syngeneic cells presenting a foreign antigen on their surface are considered to be model systems, respectively, for allograft rejection [1] and for T cell reaction against virally infected [2] or neoplastic [3] cells. The specificity of

Abbreviations used: CTL, cytotoxic T lymphocytes; T_H, T helper cells; TNBS, trinitrobenzene sulfonate; TNP, trinitrophenyl; LPS, Escherichia coli lipopolysaccharide; Con A, concanavalin A; Sn, stimulation number; FCS, fetal calf serum; TCGF, T cell growth factor, MHC, major histocompatibility complex; H-2, murine MHC; SAC, splenic adherent cells.

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the CTL is generally found associated with the serologically defined major histocompatibility complex (MHC) region products [2–4], although some CTL reactive with I region products [5] and with species-restricted self-products [6] have also been described in mouse or man. In addition to the MHC control over CTL specificity [7–9], control by an MHC region distinct from that which defines the CTL specificity has also been reported [10–12]. In order to define the cellular components which control the generation of CTL via the recognition of distinct MHC-coded products, we and others analyzed the role of distinct cell populations, such as the splenic adherent cell (SAC) [13, 14] and T helper cells (T_H) [15–18] in the generation of anti-hapten + self-CTL. Such studies indicated that an Ia^+ SAC [19] population was required, which did not influence the specificity of the generated CTL [13] and that an I-region-restricted T_H cell [18] could control the development of CTL.

The experiments to be described here were aimed at analyzing some of the antigenic and genetic parameters for the stimulation of cytotoxic T cells or T_H cells in cultures of T cells repeatedly stimulated with hapten-treated syngeneic cells. Differences appear between the specificities of the induced CTL and the specificity requirements for their stimulation whether MHC products or doses of hapten are considered. Furthermore, CTL clones derived from these cultures were found to be totally dependent on T_H cells or T-cell growth factors (TCGF) [20–21] for their growth and unresponsive to stimulation by their target antigens.

METHODS

Mice

C57BL/10 (B10), B10.BR, and recombinant mice were obtained from OLAC (Shaw Farms, Blackthorn, England) or maintained in our animal facilities. Sex-matched male or female mice, 2–5 months old, were used as donors for cell lines and stimulating cells.

Media and Culture Conditions

These were as previously described [18]. Medium RPMI-FCS, which contains RPMI 1640 plus 10 mM Hepes, 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine (Gibco), 5% heat-inactivated fetal calf serum (FCS, Eurobio, Paris, France), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) was used to initiate and maintain the cell lines, as well as to measure [^{3}H]thymidine incorporation.

Establishment of T cell Lines (18)

Briefly, mice were injected with cyclophosphamide (50 mg/kg, Endoxan, Laboratoire F., Lucien, Colombes, France) 2 days before skin sensitization with 50 μl trinitrochlorobenzene (TNCB, Polysciences, Washington, PA) 5% in acetone. Five or 6 days later, the cells obtained from the draining lymph nodes and spleen were passed over an Ig-anti-Ig column [22] with the kind help of Dr. B. Rubin. Of the T cells passed through the columns, $20–25 \times 10^6$ cells were stimulated with 20×10^6 irradiated (2000 rad from a cobalt source, Gammacell, Atomic Energy of Canada, Inc.) syngeneic spleen cells modified with the relevant concentration of hapten [7] in a final volume of 20 ml of RPMI-FCS. Cultures were in plastic culture flasks (Falcon 3013) incubated in upright position at 37°C in a water saturated atmosphere of 5% CO_2 in air. Fourteen days later (stimulation

n = 2), the primed cells were harvested and restimulated (2.5×10^6 cells) with 20×10^6 TNBS-syngeneic spleen cells. At the third (S3) and subsequent stimulations (Sn) 1×10^6 cells harvested every 9–11 days, were stimulated with 20×10^6 TNBS-treated syngeneic spleen cells. Two such T cell lines established in parallel from either B10 (line B10 (T4)) or B10.BR (line B10.BR (T2)) donor mice were studied here.

Test for T cell Proliferation

Intensity and specificity of the proliferative response were tested at the time of the restimulation of the cultures. Ten thousand primed "line" cells were stimulated in triplicate microcultures with 5×10^5 2500 rad irradiated spleen cells in a final volume of 0.2 ml in flat-bottomed wells of microtiter plates (Falcon 3034). After 2 days of culture, 2 μCi of [^{3}H]thymidine was added in each well and the cells were harvested 10 hr later and counted in a liquid scintillation counter (Intertechnique, France). Means and standard deviations of the triplicate samples were calculated.

Cytotoxic Assay

This was performed with "line" cells resuspended in RPMI 1640 × 5% FCS in V-shaped wells of microplates (Greiner). Each well contained, in a total volume of 0.2 ml, 2×10^4 ^{51}Cr-labeled (sodium chromate, New England Nuclear, Boston, MA) untreated or TNBS-treated target cells and effector cells as indicated in the tables. After a 2-min centrifugation at 400 rpm, the microplates were incubated for 4 to 5 hrs at 37°C in 5% humidified CO_2, centrifuged for 5 min at 1000 rpm, and 0.1 ml of supernatant from each well was counted in a gamma counter (Intertechnique, France). The total percentage of ^{51}Cr release was calculated for experimental and effector cells, as compared to the 100% ^{51}Cr release defined for target cells incubated in the presence of 1 N HCl. Spontaneous release for target cells in the presence of medium was subtracted from each experimental sample. Means and standard deviations of the triplicate samples were calculated. They never exceeded 5% and were left out for simplicity.

Target Cells

RDM4 (AKR/J thymoma, H-2^k), EL4 (B6 thymona, H-2^b), or blast cells obtained by culturing spleen cells for 48 hr in the presence of Escherichia coli lipopolysaccharide (LPS) (200 μg LPS/20 ml RPMI-FCS/30 × 10^6 spleen cells), or concanavalin A (Con A) (40 μg Con A/20 ml RPMI-FCS/30 × 10^6 spleen cells).

Production of Supernatant From Concanavalin A-Stimulated Rat Spleen Cells [20]

Rat spleen cells were cultured for 36 hr in the presence of Con A (300 μg Con A/100 ml RPMI-FCS 5%/2 × 10^8 spleen cells) in Falcon flasks (3024) in a horizontal position and the supernatants obtained after a 2000 rpm centrifugation were filtered through 0.45-μm sterile Millipore filters after the addition of α-methyl mannoside (10 mg/ml of supernatant). Such a supernatant is defined as 100% CASUP.

Cloning of T cells by Limiting Dilution

Forty-eight hours after the seventh restimulation of lines B10 (T4) and B10.BR (T2) 100 μl of a suspension of five line cells/ml was plated in each of the

96 wells of a flat-bottomed microtiter plate (Greiner) in a total volume of 200 μl per well containing 5×10^4 syngeneic spleen cells treated with 10 mM TNBS and irradiated at 3000 rad and 25% CASUP. After 6 and 12 days 100 μl of supernatant was aspirated from the microcultures and replaced by 100 μl of 50% CASUP. Six days after the last feeding of the microcultures, wells in which growth could be observed (less than 10% of the wells) were transferred to individual wells of Costar tissue culture plates in the presence of 2×10^6 syngeneic 10 mM TNBS-treated, 2500 rad irradiated syngeneic spleen cells and 25% CASUP and further expanded thereafter in the presence of 25% CASUP with or without TNBS-syngeneic spleen cells. One clone derived from B10.BR (T2) (61C2) and one derived from B10 (T4) (3B4) as well as subclones (61C2-14 and 3B4-10) obtained as described for the initial cloning have been used in this study.

TABLE I. Absence of Correlation Between Specificities for Stimulation of and for Lysis by T Lines Repeatedly Stimulated With TNBS-Syngeneic Cells

				Percentage of specific lysis on					
				RDM4 ($H\text{-}2^k$)			EL4 ($H\text{-}2^b$)		
Sn[a]	Responder line	Stimulating cell	E/T[b]	TNBS 10 mM	TNBS 0.1 mM	–	TNBS 10 mM	TNBS 0.1 mM	–
S4	B10 (T4)	B10 TNBS 10 mM	20	34.3	23.9	16.0	53.8	4.8	−0.1
			5.0	20.4	11.8	–	37.0	3.3	–
			1.25	7.0	2.5	–	12.0	0.7	–
	B10.BR (T2)	B10.BR TNBS 10 mM	11	49.0	38.8	8.8	2.0	1.1	0.3
			2.8	29.5	18.5	–	0.0	0.7	–
			0.68	12.9	6.3	–	−1.6	−0.8	–
S5	B10 (T4)	B10 TNBS 10 mM	4.75	15.9	–	5.0	35.4	–	−0.4
			1.20	7.3	–	–	17.4	–	–
			0.30	1.3	–	–	5.6	–	–
	B10 (T4)	B10.BR TNBS 10 mM	3.25	1.9	–	7.7	3.0	–	0.0
			0.80	0.6	–	–	1.4	–	–
			0.20	0.6	–	–	1.6	–	–
	B10.BR (T2)	B10.BR TNBS 10 mM	10.80	59.0	64.5	12.0	1.5	3.6	−0.8
			2.70	49.7	–	–	2.6	–	–
			0.67	34.7	–	–	0.8	–	–
S6	B10 (T4)	B10 TNBS 10 mM	16	36.7	–	19.0	68.1	–	7.3
			4	18.4	–	–	59.8	–	–
			1	4.4	–	–	37.0	–	–
	B10 (T4)	B10.BR TNBS 10 mM	16	0.5	–	–	18.4	–	0.4
			4	0.3	–	–	6.0	–	–
			1	0.0	–	–	1.4	–	–
	B10.BR (T2)	B10.BR TNBS 10 mM	20	59.9	–	7.3	2.6	–	5.4
			5	38.9	–	–	0.3	–	–
			1.25	16.6	–	–	2.0	–	–

[a]Number of stimulations.
[b]Effector to target cell ratio, 2×10^4 ^{51}Cr-labeled tumor cells per well.

RESULTS

Specificity Requirements for CTL Generation and for Target Cell Lysis

T cells from B10 or B10.BR mice sensitized to TNCB in vivo were restimulated in vitro with 10 mM TNBS-treated syngeneic spleen cells every 10 days as described under Methods. The established T cell lines were called B10 (T4) and B10.BR (T2), respectively. In Table I the CTL activity exhibited by these lines 4 days after their restimulation (Sn) is shown on RDM4 (H-2^k) and on EL4 (H-2^b) tumor target cells, unmodified or treated with 10 mM TNBS, or 0.1 mM TNBS. After the fourth stimulation (S4), it can be seen that the B10 (T4) line shows a cross-reactivity, its cytotoxicity being as high on H-2^b TNBS 10 mM as on H-2^k TNBS 10 mM target cells when four times more effector cells are used. The B10.BR (T2) line, on the other hand shows a rather strict restriction for H-2^k TNBS targets, whether modified with 10 or 0.1 mM TNBS. In order to analyze whether the cross-reactivity seen at the CTL level would similarly exist for the stimulation of the CTL, on stimulation 5 (S5) some B10 (T4) cells were stimulated with B10.BR-TNBS 10 mM stimulating cells and lysis was tested 3 days later on both H-2^k and H-2^b TNBS-treated target cells. The results indicate that the B10 (T4) CTL cells were very poorly stimulated by the B10.BR-TNBS stimulating cells and that no enrichment for the H-2^k-TNBS "cross-reactive" CTL has been achieved. The same conclusions can be drawn from the results obtained after the sixth stimulation (S6).

Another asymmetry between the CTL responses generated by B10 and by B10.BR spleen cells against TNBS-treated syngeneic spleen cells, which had previously been described in primary in vitro sensitization [7] was also observed in the results (S4) in Table I (S4). The lysis by the B10.BR (T2) effector cells can be detected almost as well on 0.1 mM as on 10 mM TNBS-treated H-2^k target cells,

TABLE II. Absence of Correlation Between the Dose of TNBS Required to Treat the Stimulation Cell and the Target Cell for Stimulation of and for Lysis by T Lines Repeatedly Stimulated With TNBS-Syngeneic Cells

				Percentage of specific lysis on					
				RDM4 (H-2^k)			EL4 (H-2^b)		
Sn[a]	Responder line	Stimulating cell	E/T[b]	TNBS 10 mM	TNBS 0.1 mM	–	TNBS 10 mM	TNBS 0.1 mM	–
S7	B10 (T4)	B10 TNBS 10 mM	7.4	25.9	19.3	12.8	62.2	12.7	3.3
			1.8	11.6	8.9	–	56.1	–	–
	B10 (T4)	B10 TNBS 0.1 mM	5.5	–	–	–	61.3	6.3	−3.7
	B10.BR (T2)	B10.BR TNBS 10 mM	8.2	48.8	23.9	4.1	4.5	3.8	−0.7
			2.0	19.7	13.3	–	2.8	1.7	–
	B10.BR (T2)	B10.BR TNBS 0.1 mM	6.0	22.8	7.5	−0.5	0.0	0.2	1.9
			1.5	9.2	4.1	–	0.3	−1.1	–

[a]Number of stimulations.

[b]Effector to target cell ratio, 2 × 10^4 ^{51}Cr-labeled tumor cells per well.

TABLE III. Absence of Correlation Between H-2 Region Homology Required for Stimulation of and for Lysis by T Lines Repeatedly Stimulated With TNBS-Syngeneic Cells

				Percentage of specific lysis on			
				RDM4 (H-2^k)		EL4 (H-2^b)	
Sn[a]	Responder line	Stimulating cell	E/T[b]	TNBS 10 mM	– –	TNBS 10 mM	– –
S6	B10.BR (T2)	B10.BR-TNBS 10 mM	20	59.9	7.3	2.6	5.4
			5	38.9	–	0.3	–
			1.25	16.6	–	2.0	–
	B10.BR (T2)	A.TL-TNBS 10 mM	28	59.6	2.5	3.7	–
			7	44.4	–	3.6	–
			1.75	15.8	–	−0.9	–
S7	B10.BR (T2)	B10.BR-TNBS 10 mM	8.2	48.8	4.1	4.5	−0.7
			2	19.7	–	2.8	–
	B10.BR (T2)	A.TL−TNBS 10 mM	8.4	51.0	1.2	4.2	−1.7
			2.1	28.1	–	0.9	–

[a]Number of stimulations.
[b]Effector to target cell ratio, 2×10^4 ^{51}Cr-labeled tumor cells per well.

whereas the B10 (T4) CTL lyse 10 mM but not 0.1 mM TNBS-treated H-2^b target cells. In Table II, at stimulation 7, B10 (T4) and B10.BR (T2) cells were stimulated by either 10 or 0.1 mM TNBS-treated syngeneic spleen cells and CTL activity was tested 4 days later on either 10 or 0.1 mM treated H-2^k or H-2^b tumor target cells. It can be seen that the stimulation of B10 (T4) with 0.1 mM TNBS-treated B10 stimulating cells was as efficient as that by 10 mM TNBS-treated cells but that in both cases, there was lysis of the 10 mM, but not of the 0.1 mM TNBS-treated target cells. When B10.BR (T2) cells were stimulated with 0.1 mM TNBS-treated B10.BR stimulating cells lytic activity on either 0.1 or 10 mM TNBS-treated target cells was lower than that after stimulation with 10 mM TNBS stimulating cells. These results indicate that there is no correlation between the hapten epitope density required to stimulate CTL within the T-cell lines and that required for CTL lysis of target cells.

Yet another parameter of MHC-restricted T cell stimulation is the region within the MHC which is required on TNBS-treated cells either to stimulate or to constitute a target determinant for CTL. CTL effectors from line B10.BR (T2) lysed TNBS-treated RDM4 cells (Tables I-III), which express both H-2K^k and H-2D^k products, and also B10.A (4R) (k kbbbb b b)* but not B10.MBR (b kkkkk k q) nor C3H.OH (d ddddd d k) TNBS-treated LPS blast cells, indicating that H-2 restriction of those CTL was mapping to H-2K^k (% specific lysis was respectively, 24; −3; −3 at a 1:1 effector to target cell ratio on these three LPS blast target cells). The experiments summarized in Table III indicate that A.TL TNBS-treated cells (s kkkkk k d) could stimulate the production of CTL within

*Alleles expressed at H-2 subregions are given in the order H-2K (I-A, I-B, I-J, I-E, I-C), S, D according to References [33] and [26].

TABLE IV. Stimulating Cell Requirements for the Propagation of Lines B10 (T4) and B10.BR (T2)

Stimulating cells			[³H]Thymidine incorporation by[a]	
Strain of origin	H-2[b] K I S D ABĪEC	Treatment	B10 (T4)	B10.BR (T2)
B10.BR	k kkkkk k k	–	187	1,000
		TNBS 10 mM	501	12,083[c]
		TNBS 1 mM	656	26,171
B10	b bbbbb b b	–	95	160
		TNBS 10 mM	10,637	501
		TNBS 1 mM	9,605	507
A.TL	s kkkkk k d	–	456	765
		TNBS 10 mM	751	16,738
A.TH	s sssss s d	–	72	242
		TNBS 10 mM	101	124
B10.A(4R)	k kbbbb b b	–	103	989
		TNBS 10 mM	1,424	28,755
B10.A(5R)	b bbkkd d d	–	416	479
		TNBS 10 mM	6,096	493
B10.AQR	q kkkkd d d	–	220	765
		TNBS 10 mM	395	18,659
B10.T(6R)	q qqqqq q d	–	471	330
		TNBS 10 mM	699	503

[a][³H]Thymidine incorporation as cpm/10^4 cells measured 48 hr after the ninth stimulation of the T cell lines with the indicated stimulating cells in microcultures as described under Methods.
[b]Alleles expressed at the subregions of the H-2 complex are indicated as in Reference [33].
[c]Results underlined indicate that the values are significantly different from the control +2 standard deviations.

line B10.BR (T2) after stimulations S6 or 7, although those cells could not serve as targets for the CTL (results not shown). Similarly B10 (T4) CTL appeared restricted to H-2K[b] since they lysed EL4 (no I region product), B10.A (5R) but not B10.A (4R) TNBS-treated target cells (% specific lysis was, respectively, 40 and −5 for a 3:1 effector to target ratio on these two LPS blast target cells). They could not be stimulated by B10.MBR TNBS-treated stimulating cells, however (no recovery of cells). The patterns of stimulation as measured by [³H]thymidine incorporation are indicated in the next section.

Specificity Requirements for Stimulation of Proliferation by Lines B10 (T4) and B10.BR (T2)

Table IV indicates the extent of [³H]thymidine incorporation by 10^4 cells of lines B10 (T4) or B10.BR (T2) during a 10-hr pulse, 48 hr after their stimulation by 2500 rad irradiated untreated or TNBS-treated spleen cells from mice differing in the allotypic expression of H-2 products. The results in Table IV indicate that

TABLE V. Comparison of Line B10.BR (T2) and a Derived CTL Clone (61C2) for Their Dependence on Syngeneic-TNBS Stimulating Cells or CASUP for Their Proliferation

Responding cells	Stimulating cells	Source of TCGF	[^{3}H]Thymidine incorporation after[a] 24 hr	48 hr	72 hr
B10.BR (T2)	B10.BR	–	1,549	1,421	462
	B10.BR-TNBS 10 mM	–	19,900	38,342	11,023
	–	CASUP 12.5%	20,314	31,847	35,835
	–	CASUP 25%	18,640	23,569	18,001
	–	CASUP 50%	15,658	19,465	22,952
	–	–	825	531	119
Clone 61C2	B10.BR	–	410	1,178	363
	B10.BR-TNBS 10 mM	–	753	995	232
	–	CASUP 12.5%	10,975	20,390	13,348
	–	CASUP 25%	29,848	36,786	27,830
	–	CASUP 50%	31,689	72,050	52,043
	–	–	877	852	159

[a][^{3}H]Thymidine incorporation as cpm/10^4 cells in microcultures as described under Methods.

optimal stimulation of the B10 (T4) line was obtained with either 10 or 1 mM TNBS-treated B10 spleen cells or with 10 mM TNBS-treated B10.A (5R) spleen cells expressing the b allele at K, I-A, and I-B. Stimulation with B10.A (4R) TNBS-treated cells, low but significant in this experiment, was negative in four other experiments. The B10.MBR (k^b I^k D^q) TNBS-treated cells were not capable of stimulating proliferation. These results map to I-A^b the MHC restriction for stimulation of line B10 (T4). Similarly, results in Table IV indicate that induction of proliferation of line B10.BR (T2) is optimal for TNBS-treated spleen cells expressing the I-A^k product. It should also be noted that no alloreactivity toward s, q, and k or b allelic forms of H-2 products can be detected for either B10 (T4) or B10.BR (T2) lines.

CTL Clones Derived From Lines B10 (T4) and B10.BR (T2): Their Requirements for Proliferation and Their CTL Specificity

In order to attempt to derive T cell clones with either CTL activity or T_H and/or proliferative function, cells from lines B10 (T4) and B10.BR (T2) were distributed at a concentration of 0.5 cell/well in microtiter plates containing 5×10^4 TNBS-treated 2500 rad irradiated syngeneic stimulating cells and 25% CASUP (see Methods). Cells from wells in which growth could be observed were expanded in the presence of syngeneic TNBS-treated cells and 25% CASUP. Cells were tested for proliferation requirements and for CTL activity. Table V indicates that whereas line B10.BR (T2) can be stimulated either specifically by B10.BR-TNBS cells or by CASUP in the absence of stimulating cells, clone 61C2 which expresses lysis specific for H-2K^k-TNBS (Table VI) could not be stimulated by B10.BR-TNBS cells, but could proliferate in the presence of CASUP. Whereas the extent of proliferation of clone 61c2 was proportional to the concentration of CASUP in the medium, a reverese relationship was found for the B10.BR line. The latter ef-

TABLE VI. Cytolytic Activity of Clone 61C2 Derived From Line B10.BR (T2) and 3B4-10 Derived From Line B10 (T4)

Target cells	H-2[a] K I S D A B J E C	Treatment	E/T[b]	Percentage of specific ^{51}Cr released by 61C2	3B4-10
CBA Con A blasts	k kkkkk k k	–	0.55	−2.4	−5.3
		TNBS 10 mM	0.55	31.6	−5.7
			0.13	28.0	−10.4
			0.03	27.0	−5.3
B10 Con A blasts	b bbbbb b b	–	7.5	−10	−10
		TNBS 10 mM	7.5	NT	32.4
			1.8	−2.7	21.4
			0.5	−4.6	10.9
B10.BR LPS blasts	k kkkkk k k	–	0.9	4.0	5.0
		TNBS 10 mM	0.9	24.0	8.0
B10 LPS blasts	b bbbbb b b	–	0.9	1.4	0.5
		TNBS 10 mM	0.9	9.0	26.0
B10.A(4R) LPS blasts	k kbbbb b b	–	0.9	−8.0	−4.5
		TNBS 10 mM	0.9	24.0	0.6
RDM4 tumor	k ----- k k	–	3	2.0	NT
		TNBS 10 mM	3	37.0	6.0
EL4 tumor	b ----- b b	–	3	2.0	5.0
		TNBS 10 mM	3	2.8	14.0

[a]Alleles expressed at the subregions of the H-2 complex as indicated in reference (33); (-) indicates that the corresponding H-2 product is not expressed at the surface of the tumor cells.
[b]Effector to target cell ratio; 2×10^4 ^{51}Cr-labeled target cells are present per well; spontaneous ^{51}Cr release from target cells which has been deducted to give percentage of specific ^{51}Cr release was 30–40, 30–30, 10% for Con A blasts, LPS blasts, and tumor targets, respectively.

fect may be due to a suppressive component present in the CASUP which might affect selectively a T_H and/or antigen-specific proliferative cell, but not CTL. Table VI indicates the specificity of target cell lysis by a clone (61C2) derived from line B10.BR (T2) and of a subclone (3B4.10) derived from clone 3B4 (from line B10 (T4)) after replating 0.3 cells of 3B4 per well in the presence of 1×10^5 TNBS stimulating cells and 25% CASUP. Clone 61C2 appears specific for H-2K^k + TNBS and clone 3B4.10 for H-2K^b + TNBS.

DISCUSSION

Previous work from our laboratory has shown that T cell lines could be established in vitro from in vivo TNCB sensitized T cells repeatedly stimulated in vitro with TNBS-syngeneic cells. Within such lines, cells with T_H function for the development of CTL from thymic precursors could be detected and cells with CTL function initially present were often lost after six or more restimulations [18]. The results presented here were aimed at defining the requirements for stimulation of CTL function on the one hand, and for target cell lysis by CTL on the

other hand using the same in vitro system. The parameters which have been compared at the level of CTL stimulation and that of target cell lysis by CTL are (1) H-2 haplotype (Table I); (2) TNP-hapten density (Table II) and (3) intra-H-2 region of homology (Tables III and IV). The results indicate that there is no correlation between CTL target cell lysis specificities and the requirements for restimulation of CTL within a line. For instance, if cross-reactive lysis of TNP + $H\text{-}2^k$ target cells is observed by B10 ($H\text{-}2^b$) CTL (as previously described [23]), such cross-reactive determinants present on B10.BR ($H\text{-}2^k$) TNBS cells could not be used to stimulate line B10 (T4) CTL (Table I). Precise mapping of cross-reactive determinants detected by B10 anti-B10-TNBS CTL is presently carried out at the clonal level [A. Guimezanes and A.M. Schmitt-Verhulst, in preparation].

Second, we had previously observed [7] a strict dependence for primary in vitro sensitization of CTL from B10 strain spleen cells with TNBS-treated syngeneic cells upon the epitope density of TNP on the stimulating cells, i.e., unlike B10.BR ($H\text{-}2^k$) cells, B10 cells could generate CTL only when stimulated with 10 mM but not with 0.1 mM treated TNBS-syngeneic spleen cells. Furthermore when stimulated with 10 mM TNBS-treated syngeneic spleen cells, the obtained CTL would lyse only 10 mM but not 0.1 mM treated syngeneic target cells [7]. Results presented here indicate that even after multiple in vitro restimulations, anti-syngeneic-TNBS B10 CTL lyse 10 mM but not 0.1 mM TNBS-treated target cells. However, 0.1 mM TNBS-treated syngeneic cells could be used to restimulate the CTL which retained the 10 mM TNBS requirement for target cell lysis. This observation suggests that the requirement for an epitope density on the cell surface corresponding to 10 mM TNBS is a property of the B10 CTL at the lytic phase and possibly at the initial stage of sensitization of the CTL precursors in the primary sensitization, but that for restimulation of primed CTL within the B10 (T4) line, probably via a T_H cell, a 0.1 mM TNBS epitope density is sufficient. These findings are in agreement with our results indicating that a T_H function for B-cell stimulation could be induced by 1 and 0.1 mM TNBS-treated syngeneic cells from line B10 (T4) [F. Albert, unpublished observation]. Other authors reported a difference in hapten-epitope density required for the stimulation of T_H function for B-cell stimulation when T_H cells from two noncongeneic strains of mice were compared [24].

Finally, the stimulation of the growth of lines B10 (T4) or B10.BR (T2) was found to require stimulating cells expressing TNP and an allelic form of the I-A product of H-2 homologous to that of the line cells (Table IV), whereas CTL specificity was for $H\text{-}2K^b$ + TNP and $H\text{-}2K^k$ + TNP for lines B10 (T4) and B10.BR (T2), respectively. This is in agreement with earlier findings [18, 25]. Cells from a particular recombinant (B10.MBR, (K^b I^k D^d)) mouse [26] treated with 10 mM TNBS were unable to induce proliferation and CTL restimulation in line B10 (T4) (results not shown). This suggested (a) that the determinant which was recognized by CTL ($H\text{-}2K^b$ + TNP) could not stimulate those CTL; (b) that T_H cells within the B10 (T4) line could not be stimulated by that determinant, and (c) that CTL restimulation was dependent upon the I-A + TNP dependent restimulation of a T_H cell.

In order to correlate further these specificity requirements with specific T cell functions, we attempted to derive T-cell clones from the B10 (T4) and B10.BR (T2) lines by plating limiting cell numbers in the presence of syngeneic-TNBS stimulating cells and TCGF contained in 25% CASUP [20]. Under those

conditions only CTL clones could be derived (Table VI) which were totally dependent on CASUP for their proliferation and could no longer be stimulated by TNBS-treated syngeneic cells (Table V). Since no T_H nor T cells capable of proliferating in response to antigen-specific stimulation could be obtained at the clonal level under those cloning conditions, reconstitution experiments by the addition of a cloned T_H cell line to a cloned CTL line could not be performed. Such experiments have been reported by Glasebrook et al [27] in the case of alloreactive CTL. The CTL clones derived by these authors also required TCGF for their proliferation although a responsiveness to the specific alloantigen recognized by the CTL could be detected in the presence of limiting quantities of added TCGF [27]. We were unable to detect any residual responsiveness of our CTL clones to their specific antigen even in the presence of limiting concentrations of CASUP. When more CTL clones are available after cloning under different conditions (time after in vitro stimulation, type of stimulating cells, source of TCGF), it may well appear that CTL clones with different stimulation behavior may exist. However, every CTL clone described to date is dependent on TCGF for its propagation [25]. We have recently been successful in cloning antigen-specific proliferative cells in the presence of specific stimulating cells and secondary mixed lymphocyte reaction supernatant, whereas all clones obtained from the same line in the presence of CASUP were CTL [A. Guimezanes et al, in preparation].

Such a clonal approach should help in defining the multiple sets of T cells interacting for the development of cellular and humoral immune responses and will allow to analyze separately the requirements for stimulation of each of them. It already appears that the development of a CTL response is strictly controlled by an amplifier or helper T cell compartment. This is the case (a) in ontogeny where precursors of CTL can be shown to be present early in postnatal life, but where expression of CTL activity has to wait for the maturation of T_H like cell [28]; (b) in the "immature" cortical part of the thymus where thymocytes can be shown to contain CTL precursors unable to differentiate in the absence of a T_H cell [15, 29, 30]; (c) in genetic athymic mice (Nu/Nu) from which both anti-allogeneic and anti-self-TNP CTL responses can be obtained in the presence of TCGF obtained from amplifier T cells from normal mice [29, 31, 32]. The T_H dependent behavior of the differentiated CTL which is reflected in vitro by the requirement of T_H cells or TCGF derived from such cells to maintain functional CTL in culture would suggest that every step toward production of active CTL from their precursors is highly controlled by a Lyt 1^+ 2^- amplifier T cell [29]; the question as to whether different maturational steps are controlled by the same T_H cells and their derived TCGF or whether differences might exist between the factors functioning at different stages of the differentiation of CTL precursors is presently being studied [Cooley et al, in preparation].

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