Brief Definitive Report

SELECTION OF RESTRICTION SPECIFICITIES OF VIRUS-SPECIFIC CYTOTOXIC T CELLS IN THE THYMUS: NO EVIDENCE FOR A CRUCIAL ROLE OF ANTIGEN-PRESENTING CELLS*

By ROLF M. ZINKERNAGEL

From the Institute for Pathology, University of Zurich, Universitätsspital, CH-8091 Zürich, Switzerland

Thymus-derived lymphocytes functionally express two specificities, one for major histocompatibility gene complex (MHC)-coded self major transplantation antigens and one for foreign antigenic determinants. It is still experimentally unproven whether they express one or two receptor sites (reviewed in 1–3). The restriction specificity of T cells is influenced profoundly during their differentiation in the thymus (1, 2). It is not clear whether this process involves positive selection alone or in conjunction with suppressive mechanisms. It is probable that thymic selection represents only (a crucial) part of the differentiation pathway and that post-thymic maturation is required to amplify the selected T cells (1, 3–5).

Experiments with irradiation bone-marrow chimeras or thymus transplants reconstituting thymus- and T cell-deprived mice have indicated that radioresistant (900–1,200 rad) cells in the thymus were crucially involved in determining the restriction specificity of T cells (1, 2). Thymic epithelial cells (including thymic nurse cells; 6) and antigen-presenting cells have been envoked to fulfil this function. Longo and Schwartz (7) have confirmed that early after irradiation thymic H-2 determined the restriction specificity of chimeric T cells. In addition, they presented evidence that antigen-presenting cells turn over slowly in the thymus; therefore, donor bone marrow-derived antigen-presenting cells appear only a few weeks after irradiation in the thymus and only then are capable of selecting maturing H-2-restricted antigen-specific proliferative T cells 3–5 mo after irradiation, but according to the H-2 of antigen-presenting cells rather than the thymic H-2 type. The study reported here, which uses similar and additional protocols, indicates that bone marrow-derived antigen-presenting cells are not crucially involved in thymic selection of virus-specific, MHC-restricted cytotoxic T cells.

Materials and Methods

Chimeras and Experimental Procedures. Irradiation bone marrow chimeras of $F_1 \rightarrow P$ type were produced as published previously (1, 8). Parental recipient mice were irradiated with 850–950 rad and reconstituted with T cell-depleted stem cells 1 d later. Such conventional chimeras were used for further experiments at ~16–20 wk after reconstitution. Various experimental protocols were used to deplete established chimeras of mature T cells: (*a*) Mice were treated with cortisone acetate (5 mg subcutaneously, Merck Sharp & Dohme, West Point, PA) and rabbit anti-lymphocyte serum (ALS). The ALS had been prepared by injection of 10^8 CBA or C57BL/6 thymus cells intravenously into rabbits four times in weekly intervals; the rabbits

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TABLE I

Testing of Various Protocols for T Cell Depletion In Vivo

Protocol	Treatments on days					Number of spleen cells on d6 in percent of controls	Anti-vaccinia CTL-response on day 6 in percent of controls
	−6	−4	−2	0	+2		
1	ALS	ALS	—	—	—	~100	~30
2	—	ALS	—	—	—	~130	~50
3	—	—	ALS	ALS	—	~100	~30
4	—	—	—	ALS	ALS	~100	~20
5	cortisone	ALS	ALS	—	—	~5	<5
6	ALS	ALS	cortisone	—	—	~50	~20
7	cortisone	ALS	ALS + cortisone	—	—	All animals died during vaccinia virus infection	
8	cortisone	—	—	—	—	~50	~80
9	—	—	cortisone	—	—	100	~100
10	cortisone	—	cortisone	—	—	~70	~100
	—	—	—	—	—	100	100

10 protocols were evaluated with respect to effectiveness of T cell depletion (protocols 5 and 7 were most efficient). 0.1 ml of a rabbit anti-(C57BL/6 or CBA) thymocytes (ALS) was injected intraperitoneally on days −4 and −2, 5 mg of cortisone acetate was injected in addition subcutaneously on day −6, or on day −6 and day −2. Mice did not respond to vaccinia virus with either protocol. ALS-treatment alone reduced anti-vaccinia responses about 2–5-fold, and cortisone acetate treatment alone had no significant effect.

were bled 10 d after the last boost. The result of the ALS plus cortisone treatment protocol was assessed by determining the numbers of spleen cells and the anti-vaccinia response of treated mice (Table I). Protocol 5 (cortison d-6, ALS d-4, ALS d-2) was chosen and in some cases repeated 2 mo later. Four independent groups of chimeras were treated according to this protocol. (*b*) Other chimeras were treated with cyclophosphamide (Endoxan-Asta Lot 9360, Asta-Werke AG, D-4800 Bielefeld-14, (Federal Republic of Germany) at 150 mg/kg. Four independent groups of chimeras were treated according to this protocol. (*c*) Some chimeras were irradiated a second time with three doses of 300 rad in 2-d intervals. Three groups of chimeras were treated. (*d*) A fourth group of F1 → P chimeras was irradiated a second time with a single dose of 850 rad and reconstituted again with T-cell depleted F_1 bone marrow stem cells. Three groups of chimeras were treated. Chimeras treated with either of these additional protocols (a, b, c, d) were infected 3–5 mo later and their lymphocytes tested for anti-viral cytotoxic T cell activity. Chimeric lymphocytes were H-2 typed serologically and by mixed lymphocyte culture (8). All chimeras studied were of F_1 → P type. Further experimental details are given in the Tables.

Virus Infection and ^{51}Cr Release Assay. About 10^7 plaque-forming units of WR vaccinia virus were injected intravenously into mice. 6 d later, mice were killed and spleen cells tested for anti-vaccinia cytotoxic T cell reactivity on vaccinia-virus-infected or -uninfected L929 (H-2^k), MC57G (H-2^b), or D2 (H-2^d) target cells, as described previously (8). Experimental details (duration of test, spontaneous release, etc.) are given in the Tables.

Results and Discussion

These experiments were designed to look for evidence in F_1 → P1 chimeras that antigen-presenting cells of F_1 origin populate the thymus and function to select maturing F_1 stem cells to express restriction specificity for P2. Longo and Schwartz (3) have presented evidence and argued that antigen-presenting cells of the thymus turn over more slowly than in spleens. Early on, therefore, stem cells transfused to irradiated recipients will be exposed to the original recipient type antigen-presenting cells in the chimera. When antigen-presenting cells have turned over in the thymus (at least partially) during the 6–12 wk after irradiation and reconstitution, mature T cell pools are replenished; therefore, chances for new T cells to differentiate and be selected for donor-type restriction specificities are relatively slim. One has to deplete chimeras of mature T cells that had been established 12–20 wk before to give stem cells a good chance to be selected by immigrated donor-type antigen-presenting cells

in the thymus. Several protocols were used to deplete mature T cells in chimeras at ~3–4 mo after establishment of lymphohemopoietic chimerism. In Table II, experiments are summarized with chimeras treated with cortisone plus ALS (Table II, experiment 819, 8159) or with Cytoxan (150 mg kg) (Table II, experiment 810, 8159) or with three doses of 300 rad in 2-d intervals (Table II). In all examples shown, there was no significant change of the restriction specificity pattern of treated chimeras vs. control chimeras; i.e., all experimental $F_1 \rightarrow$ P1 animals generated virus-specific cytotoxic T cells restricted to P1 but not to P2. This finding was symmetrical in $F_1 \rightarrow$ P1 and $F_1 \rightarrow$ P2 chimeras (experiment 8159). Chimeras were all H-2 typed serologically and some also by mixed lymphocyte culture; all were of F_1 type.

When chimeras were irradiated a second time with 850 rad and reconstituted again with T cell-depleted F_1 stem cells, their virus-specific effector T cells tested 12 wk after the second treatment expressed only recipient type restriction specificity (example, Table III). These doubly irradiated chimeras possessed F_1 lymphocytes by H-2 typing and by functional testing of effector T cells after anti-H-2 plus C treatment (8).

Combined, these data (from experiments using four different protocols) show that F_1 stem cells maturing in 3–5-mo-old established $F_1 \rightarrow$ P chimeras express recipient type restriction specificities predominantly. If there had been substantial numbers of antigen-presenting cells in the thymus derived from F_1 stem cells that were able to select F_1 stem cells during thymic maturation, all of the four protocols used should have allowed expression of both restriction specificities.

The results obtained cannot be explained simply by arguing that F_1 stem cells had been eliminated by the various treatments, resulting in regeneration of host-type lymphocytes and restriction specificities. All of the chimeric lymphocytes were of F_1 type, by serology and as determined by functional tests in several examples.

The experimental approach, where established chimeras were treated again with a protocol similar to that used for the induction of original chimerism, is particularly useful for discussing the role of radioresistant antigen-presenting cells vs. radioresistant thymic epithelial cells in selection of restriction specificities of cytotoxic T cells. Whatever artifacts are invoked to explain the failure of the usual $F_1 \rightarrow$ P1 chimeras to express restriction specificity for P2, they apply also to the second irradiation and reconstitution protocol.

Our results differ from those published by Longo and Schartz (7). Using the cortisone plus ALS protocol to reduce the mature T cell pool, they found that lymphocytes from $F_1 \rightarrow$ P1 chimeras expressed, with time, restriction specificity for P2. At least two possible (and testable) explanations for this discrepancy come to mind. First, because Longo and Schwartz tested T cell function and restriction in an antigen-specific proliferation assay, one may argue that induction and/or selection of restriction specificities of cytotoxic effector T cells has other requirements than that of nonlytic, differentiation-promoting T cells (helper, delayed type hypersensitivity, proliferative T cells). Differential expression of H-2 antigens might be compatible with this view, i.e., K and D are expressed more strongly on thymic epithelial cells of the cortex and subcortex, whereas Ia antigens are found in the medullary region and there predominantly on macrophagelike cells, as shown by Rouse and Weissman (9). Second, we used primary cytotoxic anti-viral T cell responses to assess restriction specificities, in contrast to the primed T cells used for antigen-specific proliferative T cell assays. There is no doubt that restriction is a quantitative phenomenon. Because

TABLE II

Experiment	Chimera	Time after reconstitution	Treatment	Test at time after treatment	Ratio of lymphocytic to target cells	Percent specific ^{51}Cr release from targets: H-2^k (L929)		H-2^b (MC57G)		H-2^d (D2.1)	
						Vaccinia	Normal	Vaccinia	Normal	Vaccinia	Normal
		wk		*wk*							
819	(CBA × C57BL/6) → C57BL/6 k × b b	20	cortisone + 2 × ALS	12	40	9	<1	62	<1		
					13	3	<1	22	<1		
					4	2	<1	16	<1		
	(CBA × C57BL/6) → C57BL/6 k × b b	20	None (control)	12	40	12	<1	64	<1		
					13	3	<1	37	<1		
					4	0	<1	13	<1		
	(BALB/c × C57BL/6) → BALB/c d × b → d	18	cortisone + 2 × ALS	10	40			3	<1	82	2
					13			<1	<1	48	<1
					4			<1	<1	28	1
	Spontaneous release (%)					18	19	19	22	26	25
810	(C57BL/6 × DBA/2) → DBA/2 b × d d	20	3 × 300 rad	12	40			<1	<1	75	<1
					13			<1	<1	42	<1
					4			<1	<1	17	<1
	(C57BL/6 × DBA/2) → DBA/2 b × d d	20	Cytoxan	12	40			<1	<1	83	<1
					13			<1	<1	43	<1
					4			<1	<1	38	<1
	(C57BL/6 × DBA/2) → DBA/2 b × d d	20	None (control)	12	40			<1	<1	48	<1
					13			<1	<1	22	<1
					4			<1	<1	13	<1
	Spontaneous release (%)							23	19	17	18
8159	(C57BL/6 × DBA/2) → DBA/2 b × d d	30	cortisone + 2 × ALS	18	40			<1	<1	54	<1
					13			<1	<1	17	<1
					4			<1	<1	8	<1
			Cytoxan	18	40			<1	<1	60	<1
					13			3	<1	34	<1
					4			<1	<1	25	<1
			None (control)	18	40			2	<1	57	<1
					13			<1	<1	40	<1
					4			<1	<1	26	<1
	(C57BL/6 × DBA/2) → C57BL/6	30	cortisone + 2 × ALS	18	40			78	<1	17	<1
					13			24	<1	<1	<1
					4			14	<1	6	<1
			Cytoxan	18	40			62	<1	17	<1
					13			39	<1	29	<1
					4			17	<1	2	<1
			None (control)	18	40			55	<1	7	<1
					13			32	<1	<1	<1
					4			9	<1	13	<1
	Spontaneous release (%)							25	20	22	19

TABLE III

Experimental	Chimera (H-2 type)	Time after reconstitution	Treatment	Test at time after treatment	Ratio of lymphocytic to target cells	Percent specific ^{51}Cr release from targets: H-2^k (L929)		H-2^b (MC57G)		H-2^d (D2.1)	
						Vaccinia	Normal	Vaccinia	Normal	Vaccinia	Normal
		wk		*wk*							
8170	(C57BL/10 × B10.BR) → B10.BR (b × k → k)	16	850 rad + 2 × 10^7 anti-θ + C	12	40	41	3	6	3		
					13	21	8	3	<1		
			Bone marrow cells		4	6	3	2	<1		
	(C57BL/10 × B10.BR) → B10.BR (b × k → k)	16	None control	12	40	51	2	7	2		
					13	25	1	1	<1		
					4	10	2	2	<1		
	Control B10.BR (k)				40	43	2	3	3		
					13	20	2	1	<1		
					4	8	1	2	<1		
	C57BL/10 (b)				40	<1	<1	42	<1		
					13	<1	<1	29	2		
					4	<1	<1	11	1		
	Spontaneous release (%)					22	35	16	20		

Chimeras were irradiated with 875 or 900 rad and reconstituted with 1-2 × 10^7 anti-θ + C-treated bone marrow cells. At the indicated time thereafter, they were treated as shown and tested after the interval shown. *Test duration*, 6 h; all chimeras were H-2 types; >90% of the spleen cells were of F_1 origin.

in primary anti-vaccinia experiments activity on infected recipient-type targets is at least 10–30 times greater than the (in most cases unmeasurable) response on nonhost P2 targets, our results derive from the sensitive part of the titration curve. Titrations of proliferative T cell activities may be needed to assess whether there are no quantitative differences with respect to T cells restricted to P1 vs. P2.

Summary

The proposal was tested that (P1 × P2) $F_1 \rightarrow$ P1 irradiation bone marrow chimeras expressed predominantly P1-restricted T cells because donor derived stem cells were exposed to recipient derived antigen-presenting cells in the thymus. Because P1 recipient-derived antigen-presenting cells are replaced only slowly after 6–8 wk by (P1 × P2) donor-derived antigen-presenting cells in the thymus and because replenished pools of mature T cells may by then prevent substantial numbers of P2-restricted T cells to be generated, a large portion of thymus cells and mature T cells were eliminated using the following treatments of 12–20-wk-old (P1 × P2) $F_1 \rightarrow$ P1 irradiation bone marrow chimeras: (*a*) cortisone plus antilymphocyte serum, (*b*) Cytoxan, (*c*) three doses of sublethal irradiation (300 rad) 2 d apart, and (*d*) lethal irradiation (850 rad) and reconstitution with T cell-depleted (P1 × P2) F_1 stem cells. 12–20 wk after this second treatment, (P1 × P2) → P1 chimeras were infected with vaccinia-virus. Virus-specific cytotoxic T cell reactivity was expressed by chimeric T cells of (P1 × P2) F_1 origin and was restricted predominantly to P1. Virus-specific cytotoxic T cells, therefore, do not seem to be selected to measurable extent by the immigrating donor-derived antigen-presenting cells in the thymus; their selection depends apparently from the recipient-derived radioresistant thymus cells.

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