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Cell-Mediated Immune Responses in vitro: Interaction of Thymus-Derived Cells during Cytotoxic Allograft Responses in vitro

Abstract. *A mouse in vitro allograft system was used to check for synergism among thymus-derived cells during cell-mediated immune responses. Synergistic interactions occurred between thymus and thymus-derived lymphocytes. The majority of precursors of cytotoxic effector cells were peripheral thymus-derived cells; thymocytes acted mainly as amplifier cells.*

Both murine thymocytes and thymus-derived (T) lymphocytes have been shown to be capable of mounting cytotoxic allograft responses in vivo (*1*) and in vitro (*2*), apparently without requiring collaboration with bone marrow (B)–derived lymphocytes. When compared on a cell to cell basis, thymocytes were found to be poor precursor cells of cytotoxic lymphocytes (CL) in vitro in contrast to recirculating T cells, splenic T cells being intermediate (*2*). These findings suggested that there exists a heterogeneity within thymus-processed lymphocytes in their capacity to differentiate in vitro into cytotoxic effector cells. Indeed evidence for the existence of functional T cell subpopulations has appeared (*3, 4*). For example, Cantor and Asofsky (*4*) proposed a model for the generation of thymus-derived effector cells mediating graft-versus-host reactions, in which two distinct types of T cells appear to be involved: one acting as an amplifier cell and the other type functioning as the precursor of the effector cell. I have now tested the concept of T-T cell interactions during cell-mediating immune responses in an in vitro allograft system.

About 15×10^6 viable dissociated CBA/H/Wehi (H-2^k) or AKR/J (H-2^k) mouse thymocytes or inguinal lymph node (LN) cells, or a mixture of both, were cultured together with 2×10^6 mitomycin C–treated BALB/c spleen cells (H-2^d) for 6 days in modified Eagle's medium containing 5 percent fetal calf serum in the Marbrook-Diener culture system (*2, 5*). Cells from three cultures per group were pooled, washed twice, and assayed for in vitro generated cytotoxic activity in a modification (*2*) of the ^{51}Cr release assay described by Brunner *et al.* (*6*). Cytotoxic activity generated in vitro was compared on a culture basis (*7*). Thus, the number of lymphocytes at the initiation of the culture was kept identical in the various groups of cultures, and the cytotoxic activity generated was compared relative to that obtained with LN cells as precursor cells of cytotoxic lymphocytes. In actual fact, cytotoxic lymphocytes harvested from different groups were assayed for cytotoxic activity at the cell concentration which resulted (in the case of LN derived cytotoxic lymphocytes) in a ratio of cytotoxic lymphocytes to target cells of 12 to 1, 2 to 1, and 0.3 to 1. The DBA/2 derived mastocytoma cell line P815 (*8*), cultured in vitro and of identical H-2 specificity as BALB/c spleen cells used for immunization, was used as target cells in the ^{51}Cr release assay. AKR antiserum to CBA θ antigen was prepared according to Raff (*9*) and used as described (*7*). The antiserum killed 80 percent of CBA thoracic duct lymphocytes in the presence of agarose-absorbed guinea pig serum, and the activity against θ could be absorbed by brain from CBA mice. Mitomycin C treatment was administered at a final concentration of 40 μg/ml for 30 minutes (*2*). Irradiation (900 rads) of dispersed lymphocytes was performed as described by Miller and Sprent (*10*).

As given in Table 1, CBA LN cells (an example of a lymphocyte population containing 80 percent of peripheral T cells) represented a good precursor cell population for cytotoxic lymphocytes. Thus, after a 6-day culture period in the presence of cell-bound alloantigen (2×10^6 mitomycin C–treated BALB/c spleen cells), cytotoxic activity was generated which lysed 80 percent of the ^{51}Cr-labeled P815 target cells at a ratio of cytotoxic lymphocytes to target cells of 2 to 1 within 200 minutes. That the reacting cells were T lymphocytes was suggested by the fact that treatment of LN cells before culture with AKR antiserum to θ antigen, plus complement, abolished the capacity of the cells to mount a cytotoxic

Table 1. Synergistic effect of a mixture of thymocytes plus syngeneic lymph node cells in cytotoxic allograft responses in vitro.

Test system	Lysis (%)* of ^{51}Cr-labeled P815 target cells (H-2^d) at a CL to target cells ratio of:		
	12 : 1	2 : 1	0.3 : 1
15×10^6 CBA LN	100	80	40
15×10^6 CBA LN, treated with antiserum to θ	13	12	12
15×10^6 CBA thymocytes	30	14	12
1.5×10^6 CBA LN plus 14×10^6 CBA irradiated thymocytes	25	13	12
1.5×10^6 CBA LN plus 14×10^6 CBA thymocytes	100	91	52
1.5×10^6 (CBA LN), treated with antiserum to θ plus 14×10^6 CBA thymocytes	25	14	12
1.5×10^6 (CBA $\times$ BALB/c) F_1 LN plus 14×10^6 CBA thymocytes	23	13	12
1.5×10^6 CBA LN plus 14×10^6 (CBA $\times$ BALB/c) F_1 thymocytes	38	27	13
1.5×10^6 (CBA LN) mitomycin C–treated plus 14×10^6 CBA thymocytes	24	12	12

* Responder cells (H-2^k) were cultured together with 2×10^6 mitomycin C–treated allogeneic BALB/c (H-2^d) spleen cells. Cytotoxic activity generated in vitro was assayed in a ^{51}Cr test for 200 minutes. Cytotoxicity obtained was compared on a culture basis relative to the cytotoxicity generated by CBA LN cell derived cytotoxic lymphocytes. Background lysis of P815 target cells (in the presence of normal lymphocytes) was 12 ± 1.3 percent. Similar results were obtained in 12 independent experiments.

allograft response in vitro. In contrast to LN cells, CBA thymocytes were far less effective in differentiating in vitro into cytotoxic effector cells. CBA thymocytes (15×10^6 per culture) yielded cytotoxic lymphocytes able to lyse only 30 percent of the target cells. A similar degree of cytotoxicity was obtained with 1.5×10^6 LN cells in the presence of 14×10^6 irradiated thymocytes. (1.5×10^6 LN cells in the presence of 14×10^6 nonirradiated syngeneic thymocytes, obtained from the dense cell fractions of a continuous albumin gradient, also resulted in only 35 percent lysis when cultured in the presence of cell-bound alloantigen; unpublished results.) However, when a mixture of 1.5×10^6 LN cells and 14×10^6 normal thymocytes were cultured together with cell-bound alloantigen, the magnitude of cytotoxic response obtained was significantly greater than the sum of activity obtained with thymocytes and LN cells cultured alone. In actual fact, the cytotoxic activity generated by a mixture of 14×10^6 thymocytes and 1.5×10^6 LN cells equaled or exceeded that obtained with 15×10^6 LN cells (Table 1). Similar types of synergy could be demonstrated with mixtures of thymocytes and peripheral blood cells or thoracic duct lymphocytes, and it was noted that as few as 3×10^6 to 5×10^6 thymocytes plus 1.5×10^6 LN cells still produced the synergistic phenomena described. The synergism observed between thymocytes and syngeneic peripheral T cells appeared to be dependent on the capacity of both cell populations to recognize alloantigen. Thus thymocytes or T cells derived from F_1 (CBA $\times$ BALB/c) hybrids were ineffective, and treatment of T cells or thymocytes with mitomycin C abolished the synergistic effect observed.

In order to evaluate the relative contribution of the 14×10^6 thymocytes and 1.5×10^6 peripheral T cells to the final cytotoxic activity generated, a cell marker that allowed the identification of thymus or peripheral T cell derived cytotoxic lymphocytes was necessary. Since CBA and AKR mice are identical in regard to H2 specificities (*11*) but differ in regard to the θ antigen phenotype (*12*), the possibility of inducing synergy in cytotoxic allograft responses with AKR thymocytes and CBA LN cells was tested. In this cell combination, it was thought possible to abolish the CBA LN derived cytotoxic lymphocytes by treatment with AKR antiserum to CBA θ antigen, plus complement. The remaining cytotoxic activity could then be identified as being derived from AKR thymocytes. Table 2 shows that synergy among T cells in in vitro allograft responses occurred with a combination of AKR thymocytes and CBA LN cells. Table 2 illustrates also that unlike the cytotoxic activity generated in a mixture of CBA thymocytes and CBA LN cells which could be abrogated by treatment with AKR antiserum to θ, plus complement, such treatment did not alter significantly AKR LN cytotoxic activity derived from AKR LN cells. However, treatment with AKR antiserum to θ, plus complement, reduced the cytotoxic activity generated in a mixture of AKR thymocytes (14×10^6) and CBA LN cells (1.5×10^6) from 81 percent lysis to 24 percent (ratio of 2:1). Since 1.5×10^6 reactive CBA LN cells alone yielded poor cytotoxic activity—even though high cytotoxic activity was generated in the presence of AKR thymocytes (a great deal of which was abolished after treatment with AKR antiserum to θ, plus complement)—these findings suggested that the responsiveness of peripheral T cells was very much enhanced in the presence of responding thymocytes. On the other hand, also, the responsiveness of thymocytes was enhanced in a mixture of thymocytes plus LN cells. A quantitation of the respective contribution of thymocytes and LN cells to the overall cytotoxic response revealed that about 70 percent was due to the increased response of peripheral T cells, the remaining activity being due to the increased response of thymocytes. Similar conclusions were drawn on the basis of reciprocal experiments in which the cytotoxic response obtained in a mixture of AKR LN cells and CBA thymocytes was analyzed.

Table 2. Synergism among AKR thymocytes and CBA LN cells and evaluation of the respective contribution to the cytoxic activity generated; N.D., not done; C, complement.

Test system	Lysis (%)* of ^{51}Cr-labeled P815 target cells (H-2^d) at a CL to target cells ratio of:		
	12 : 1	2 : 1	0.3 : 1
15×10^6 AKR LN cells†	100	96	47
Residual activity after AKR serum treatment plus C	100	89	40
Residual activity after AKR antiserum to θ treatment plus C	100	88	38
15×10^6 AKR thymocytes	33	16	N.D.
15×10^6 CBA thymocytes	41	19	N.D.
1.5×10^6 CBA LN cells plus 14×10^6 AKR irradiated thymocytes	18	11	N.D.
1.5×10^6 CBA LN cells plus 14×10^6 AKR thymocytes†	100	94	50
Residual activity after AKR serum treatment plus C	100	81	36
Residual activity after AKR treatment with antiserum to θ plus C	54	22	12
1.5×10^6 CBA LN cells plus 14×10^6 CBA thymocytes†	100	75	31
Residual activity after AKR serum treatment plus C	100	72	22
Residual activity after AKR treatment with antiserum to θ plus C	11	12	11

* Responder cells (H-2^k) were cultured together with 2×10^6 mitomycin C–treated allogeneic BALB/c (H-2^d) spleen cells. After 6 days in culture, cells per group were harvested. † Nine cultures were set up instead of three. Each cell was divided into three equal parts. One part remained untreated, one part was treated with normal AKR serum plus complement, and one part was treated with AKR antiserum to CBA θ antigen plus complement. Cytotoxic activity of each cell pool was then assayed in a ^{51}Cr test for 200 minutes and compared to the cytotoxic activity of AKR LN derived cytotoxic lymphocytes. Background lysis of P815 target cells (in the presence of normal lymphocytes) was 11 ± 2.1 percent.

At present, the nature of the cooperative cell interaction between thymocytes and peripheral T cells in cytotoxic allograft responses in vitro is unknown. The results are reminiscent of those reported by Cantor and Asofsky in studies of the cells that initiate graft-versus-host responses in F_1 neonatal mice (*4*). In an elegant hypothesis, these authors suggested that different maturation stages of T lymphocytes exist (termed T_1 and T_2) and that cell collaboration between T_1 (precursor cells of effector cells) and T_2 (amplification cells) takes place (*4, 13*). Further experiments are needed in order to define the reactive T cell subpopulations that cooperate in cell-mediated immune responses in vitro, and to test the hypothesis that different "antigens" on the cell surface of the stimulating cells are responsible for the synergistic interaction between thymocytes and peripheral T cells during cytotoxic allograft responses in vitro.

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Subfornical Organ: Site of Drinking Elicitation by Angiotensin II

Abstract. *Angiotensin II applied directly to the subfornical organ in a dose as small as 0.1 nanogram elicited short-latency drinking behavior in water-sated rats. Lesions in the body of this structure blocked drinking induced by angiotensin II applied to the basal telencephalon (including preoptic area). These results call attention to the subfornical organ as an important central nervous structure involved in the conrol of drinking behavior.*

The renin-angiotensin system, known for its pressor effects mediated through angiotensin II, has been implicated in processes of body fluid regulation. Hypovolemia, a decrease in the absolute volume of the body fluids, is one of several stimuli now thought to increase renin secretion from the juxtaglomerular cells of the kidney (*1*). Circulating renin catalyzes the conversion of the serum α_2 globulin peptide angiotensinogen into angiotensin I, and this product is transformed by circulating angiotensin-converting enzymes into the biologically active angiotensin II (*2*). A renal dipsogenic factor (that is, a factor eliciting drinking) thought to be renin has been identified (*1*), and direct intracranial injection of angiotensin II produces drinking in water-sated animals, a result suggesting a central nervous system site of action of angiotensin II (*3*).

Autoradiographic analysis of the penetration of angiotensin II into the brain has shown that the octapeptide reaches equilibrium slowly with brain interstitial fluid (*4*). It therefore seemed plausible that a central dipsogenic receptor for angiotensin II would lie outside the blood-brain barrier. Evidence from our laboratory had implicated the subfornical organ (SFO) as a major site of action of the dipsogenic cholinomimetic, carbachol, and this structure may have a significant role in body fluid regulation processes (*5*, *6*). Anatomical and histochemical evidence also suggested that this structure lies outside the blood-brain barrier (*7*). We therefore tested the dipsogenic effect of angiotensin II applied directly to the SFO. Because intracranially injected chemicals can also spread via the ventricles (*8*) or the vasculature (*9*), we also studied the consequences of SFO lesions on drinking produced by application of angiotensin II to the preoptic area, which Epstein *et al.* (*10*) reported to be the most effective central locus in eliciting drinking behavior.

In the first experiment, we performed dose-response and time course analyses of the drinking behavior elicited by angiotensin II applied directly to the SFO. The methods have been described (*6*). Adult male albino rats, weighing 300 to 400 g, were each implanted with a single 27-gauge guide cannula aimed at the SFO by stereotaxic methods (*11*). Animals were permitted to recover from surgery for 3 days with free access to food and water. On days 4 to 7 after surgery, baseline measurements of food and water intake were taken once daily for a 0.5-hour period. Mean food and water intakes during these 4 days were defined as baselines, and these values were subtracted from those after intracranial chemical injections to give the net intake values shown in Fig. 1. On day 8 each animal received a single 0.5-μl intracranial injection of angiotensin II solution (Hypertensin, Ciba) delivered in an isosmotic saline vehicle (*12*) at 0.1 μl/sec. Food and water intakes were monitored for the 0.5-hour period after the injection as they had been during the preceding 4 days of baseline measurements. Within 6 hours after completion of testing, animals were killed with an overdose of pentobarbital; brain tissue was fixed in situ by intracardiac aldehyde perfusion. Alternating 30-μm frozen sections through the cannula tract in each brain were stained for myelin (Weil method) or for Nissl substance (thionin method).

The first experiment demonstrated that the SFO was remarkably sensitive to the dipsogenic effect of intracranially administered angiotensin II. As shown in Fig. 1a, angiotensin II applied to the SFO in doses ranging from 0.1 to 100 ng elicited a drinking response. As little as 0.1 ng of angiotensin II in the SFO elicited a drinking response in a group of five animals; their water intake for the first 15 minutes after injection was more than the mean baseline intake for a similar period ($t = 3.061$, d.f. $= 4$, $P < .05$).

Epstein *et al.* (*10*), using similar intracranial injection methods but not injecting into the SFO, reported that the brain area most responsive to the dipsogenic effect of angiotensin II was the medial preoptic area, and that 5 ng was the lowest effective dose. Our effective dose of 0.1 ng is more than 0.5 log unit less than that reported by Epstein *et al.* Our data suggest, therefore, that the SFO is a more effective site than the medial preoptic area for eliciting angiotensin-induced drinking. In addition, we believe this is the lowest reported dose of a chemical applied to the brain which elicits a behavioral response.

Analysis of the time course of the elicited water intake (Fig. 1b) supported the view that the SFO may be an important site of action of angiotensin II since, at all doses, drinking behavior began rapidly, usually in less than 30 seconds. The duration of the elicited drinking behavior varied with dose: larger doses of angiotensin II (10 to 100 ng) elicited bursts of intense drinking behavior lasting 10 to 15 minutes, whereas lower doses (0.1 to 0.5 ng) elicited 1- to 3-minute drinking bursts. The rapid onset of drinking observed with doses of angiotensin II applied to the SFO is consistent with the view that the dipsogen is acting at this central nervous system site.