

Effect of Human Thymic Epithelial Conditioned Medium on *in Vitro* and *in Vivo* Alloantigen-Induced Lymphocyte Activation in the Mouse

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Human thymic epithelial conditioned medium (HTECM), but not control conditioned media, has been shown previously to enhance the Con A and PHA responsiveness of human and mouse thymocytes and the PHA response of spleen cells. In this study we have demonstrated that HTECM increased the reactivity of mouse thymocytes in one-way mixed lymphocyte culture and in cell-mediated cytotoxicity, while no enhancing effects were observed with spleen cells. Incubation of mouse thymocytes with HTECM rendered the cells more reactive in *in vivo* graft versus host reactions. The findings indicate that HTECM, an *in vitro* produced factor of the human thymus, plays an important role in the induction of immunocompetence in thymocytes.

INTRODUCTION

The thymus contains lymphoid cell populations of various stages of maturity. The majority of these thymocytes is immature T lymphocytes with low immunocompetence (1). Humoral factors produced within the thymus environment can modulate the expression of surface antigens and immunocompetence of thymocytes (2) by interaction with the immature thymocyte subpopulations (2). These humoral factors are probably produced by thymic epithelial cells (3-6). Recently we have described an enhancement of the response of human and mouse thymocytes to T mitogens by supernatant of human thymic epithelial cell cultures (HTECM) (7).

In this study we investigated the effects of HTECM on alloantigen-induced lymphocyte proliferation and on T-cell effector functions in the mouse *in vitro* and *in vivo* (respectively, cell mediated cytotoxicity (CMC) and graft versus host reactivity). The mixed lymphocyte culture (MLC) is a model which represents the recognition phase of the *in vivo* graft versus host (GvH) reaction. The response of thymocytes stimulated in MLC is rather low. In addition, only a few alloantigen-stimulated thymocytes develop into cytotoxic effector cells, and the few precursor killer cells are found only in the cortisone-resistant population (8, 9). The capacity of lymphocytes to induce a GvH response is a measure of cell-mediated immunocompetence *in vivo* (10).

MATERIALS AND METHODS

Human thymic epithelial cultures and control cultures. Human thymuses were obtained from children undergoing cardiac surgery. Thymic epithelial cells were

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cultured from tissue fragments in Hepes (25 mM)-buffered RPMI-1640 medium (H-RPMI) with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μ g/ml), and 20% heat-inactivated human AB serum: This procedure as well as the collection of conditioned media were performed as described previously (7). Conditioned media from human labial epithelial cultures and amnion epithelial cultures were used as controls. All control conditioned media showed the same effect; therefore, only the results obtained with human labial epithelial conditioned medium (HLECM) are shown in the figures and tables.

Mice. Five- to ten-week-old female Swiss (H-2^s), Balb/c (H-2^d), DBA/2 (H-2^d), and C57Bl/6 (H-2^b) mice were used in all experiments.

Mixed lymphocyte culture (MLC). Cultures were prepared fivefold in round-bottom microtiter plates (M24-ARTL, Greiner, Nürtingen, FRG). Each well contained 0.15 ml of Hepes-buffered RPMI-1640 with bicarbonate, L-glutamine, antibiotics, and 10% heat-inactivated human AB serum. One $\times 10^6$ Swiss responder thymocytes were cultured together with 0.5×10^6 stimulator spleen cells, irradiated with 3000 rad from a Philips RT 250 with a HVL of 2.05 mm Cu immediately prior to use, and diluted conditioned medium. Responder spleen cells were used at a concentration of 0.5×10^6 per well. Cultures were maintained for 4 days at 37°C in a humidified atmosphere with 5% CO₂. Sixteen hours before the end of the culture period, 1 μ Ci [methyl-³H]thymidine (5 Ci/mmol; The Radiochemical Centre, Amersham, U.K.) was added to each well. The cells were collected on Titertek glass-fiber filters with the aid of a multiple cell culture harvester (Titertek, Skatron A/S, Lierbyen, Norway). The radioactivity was measured with a Nuclear Chicago liquid scintillation counter and the results are expressed as the mean cpm \pm standard error (SE).

Cell-mediated cytotoxicity (CMC). MLC was performed as described previously in medium containing 5×10^{-5} M mercaptoethanol. The cultures were then incubated at 37°C for 5 days.

P-815, a DBA/2-derived mastocytoma cell line, and EL-4, a C67Bl/6-derived lymphoma cell line, were used as target cells in the cytotoxicity assays. The tumor cell lines were maintained by weekly passage in DBA/2 and C57Bl/6 mice, respectively, and used 4 to 5 days after passage. The tumor cells (1×10^7) were incubated in 1 ml of H-RPMI containing 10% AB serum and 200 μ Ci of ⁵¹Cr-labeled sodium chromate (100–350 μ Ci/ μ g Cr; The Radiochemical Centre, Amersham, U.K.) for 0.5 hr at 37°C. The cells were then washed three times with H-RPMI without serum and once with H-RPMI containing 10% AB serum. Two $\times 10^4$ ⁵¹Cr-labeled target cells in a volume of 0.05 ml were added to each well of the MLC as described by Simon *et al.* (11). The culture plates were centrifuged at 100g for 1 min and thereafter incubated for 4.5 hr under the same conditions as described previously. The plates were then centrifuged at 250g for 10 min, and a 100- μ l sample of the supernatant fluid from the top of each well was collected. Otherwise, supernatant fluids were collected with a supernatant collection system (Titertek).

The radioactivity was counted in a Baird Atomic gamma counter. The results of the cytotoxicity assay are expressed as percentage specific ⁵¹Cr release following the formula:

$$\frac{\text{cpm } ^{51}\text{Cr release in allogeneic cultures} - \text{cpm } ^{51}\text{Cr release in syngeneic cultures}}{\text{cpm maximum } ^{51}\text{Cr release} - \text{cpm spontaneous } ^{51}\text{Cr release}} \times 100\%.$$

The maximum ^{51}Cr release value was determined by the addition of 0.15 ml of 5% Triton X-100 to 2×10^4 labeled target cells. Spontaneous release in the presence of medium was not significantly different from that obtained in syngeneic cultures and was always less than 20%.

Graft versus host (GvH) assay in vivo. Swiss thymocytes were washed in H-RPMI and resuspended at a concentration of $20-30 \times 10^6/\text{ml}$. Cells were incubated for 2 hr at 37°C with HTECM, HLECM, or tissue culture medium at a dilution of 1:15. The cells were washed, then resuspended in H-RPMI, and 10^7 viable cells in 0.1 ml were injected i.p. into neonatal Balb/c hosts (three or four per group). One or two mice in each litter received 0.1 ml of H-RPMI. The neonatal Balb/c mice were injected with the cell suspensions within 24 hr after birth. Nine days later splenomegaly (mg spleen weight/g body weight) was determined (10). A spleen index greater than 1.3 is usually regarded as a positive GvH response (10). Calculations of mean values of the spleen index and standard error (SE) were made on a geometric basis as recommended (12).

Spontaneous uptake of tritiated thymidine. Splenomegaly in GvH reactions is partly due to lymphocyte proliferation after alloantigen recognition (10), as can be measured *in vitro* in MLC reactions. In some experiments an estimate of the lymphocyte proliferation *in vivo* was obtained by spontaneous $[^3\text{H}]\text{TdR}$ incorporation in spleen cells *in vitro*. 2×10^5 spleen cells from mice used in the GvH test were cultured in 0.15 ml of MLC culture medium for 4 hr at 37°C in the continuous presence of $1 \mu\text{Ci } [^3\text{H}]\text{TdR}$. Thereafter the cultures were harvested and radioactivity measured as described previously.

Statistical methods. Statistical analysis of the data was performed on a Tektronic-31 apparatus using Student's *t* test.

RESULTS

Effect of conditioned media on the MLC reactivity of mouse lymphocytes. The $[^3\text{H}]\text{TdR}$ incorporation into alloantigen-stimulated mouse thymocytes was markedly enhanced by the addition of HTECM as compared with control conditioned media (Fig. 1). HTECM had no effect on the MLC reactivity of the syngeneic cell cultures. The peak of activity of HTECM was usually observed at a dilution of 1:3 to 1:6. HTECM had no effect on the one-way MLC of spleen cells (Table 1).

To induce cytotoxic T cells, experiments were carried out in medium supplemented with mercaptoethanol (ME). Therefore experiments were performed to ascertain whether the addition of ME could influence the HTECM effect in MLC. As seen in Table 2, MLC reactivity of thymocytes in medium with ME is higher than in cultures without ME, but also in this experiment an increase in thymidine incorporation by HTECM is noted.

Effect of HTECM on the generation of cytotoxic T lymphocytes. Thymocytes from Swiss mice (H-2^s) were cultured with Balb/c (H-2^d) stimulator spleen cells in

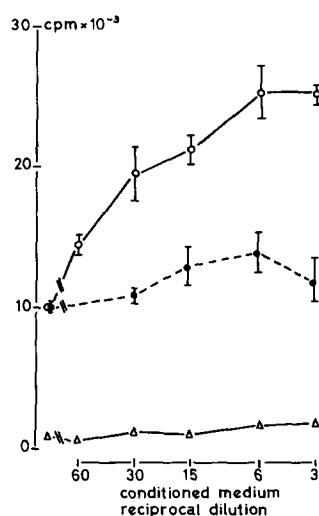


FIG. 1. Effect of various dilutions of HTECM (—) and HLECM (---) on [^3H]TdR incorporation into Swiss thymocytes stimulated with irradiated allogeneic (Balb/c) (\circ , \bullet) and syngeneic (Δ) spleen cells. Symbols represent the mean cpm \pm SE of quintuple cultures containing 1×10^6 responder cells and 0.5×10^6 stimulator cells. [^3H]TdR incorporation into syngeneic cultures was not affected by the addition of HTECM. The SE is shown only when it exceeded the size of the symbols. The results are representative of five separate experiments.

TABLE 1
EFFECT OF HTECM ON THE RESPONDER ACTIVITY OF MOUSE THYMOCYTES AND
SPLEEN CELLS IN ONE-WAY MLC

Responder cells ^a	Addition	Allogeneic	<i>P</i>	Syngeneic
Thymocytes	Plain medium	5,372 \pm 949 ^b		433 \pm 40
	HLECM (1/15) ^c	5,641 \pm 300	n.s.	390 \pm 17
	HTECM (1/15)	10,285 \pm 1,302	<0.01	425 \pm 39
Spleen cells	Plain medium	117,100 \pm 3,096		51,422 \pm 1,477
	HTECM (1/15)	102,732 \pm 2,152		43,068 \pm 2,869

^a Cell concentrations used: responder Swiss thymocytes, 1×10^6 /culture; responder spleen cells, 0.5×10^6 /culture; and irradiated allogeneic (Balb/c) and syngeneic spleen cells, 0.5×10^6 /culture.

^b Results are expressed as cpm \pm SE and are representative of five separate experiments.

^c Final dilution of conditioned medium.

TABLE 2
EFFECT OF HTECM ON THE RESPONDER CAPACITY OF MOUSE THYMOCYTES IN ONE-WAY MLC
IN THE PRESENCE OF MERCAPTOETHANOL^a

Addition	Allogeneic	<i>P</i>	Syngeneic
Plain medium	17,619 \pm 2612		490 \pm 62
HLECM (1/15)	14,056 \pm 1872	n.s.	849 \pm 130
HTECM (1/15)	36,612 \pm 4015	<0.0025	554 \pm 152

^a The conditions were identical to those for Table 1, with the exception that 5×10^{-5} M mercaptoethanol was added to the culture medium. Results are representative of three separate experiments.

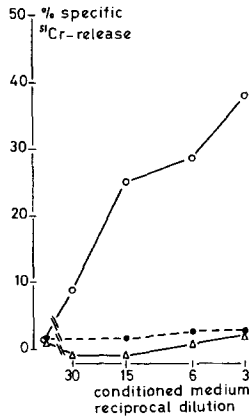


FIG. 2. Effect of various dilutions of HTECM (—) and HLECM (---) on the cytotoxic response of mouse thymocytes (H-2^b) after stimulation with irradiated spleen cells (H-2^d). Cytotoxicity was measured against 2×10^4 ⁵¹Cr-labeled P-815 (H-2^d) (○, ●) or EL-4 (H-2^b) (△) cells and expressed as percentage specific ⁵¹Cr release; for formula, see Materials and Methods. Figures are representative of three separate experiments. The cytotoxic response of spleen cells ranged from 60 to 80%. Cell concentrations were the same as in Fig. 1.

a 5-day MLC. The specific ⁵¹Cr release after the addition of ⁵¹Cr-labeled P-815 cells (H-2^d) is shown in Fig. 2. The addition of HTECM to the MLC resulted in a marked increase in the cytotoxic response. No cytotoxicity was measured against ⁵¹Cr-labeled EL-4 cells (H-2^b).

Table 3 shows the results when spleen cells were used as responder cells. Spleen cells gave a much stronger cytotoxic response than thymocytes: No effect of HTECM on this response was noted.

Effect of HTECM on the GvH reactivity of thymocytes. One $\times 10^7$ viable Swiss

TABLE 3
EFFECT OF HTECM ON THE CYTOTOXIC RESPONSE OF MOUSE THYMOCYTES AND
SPLEEN CELLS STIMULATED IN ONE-WAY MLC

Effector cells ^a	Addition	% Specific ⁵¹ Cr release ^b		
		Expt 1	Expt 2	Expt 3
Thymocytes	Plain medium	4	2	2
	HLECM (1/6)	—	—	3
	HLECM (1/15)	7	0	2
	HTECM (1/6)	—	—	29
	HTECM (1/15)	22	14	24
	Plain medium	57	52	80
Spleen cells	HLECM (1/6)	—	—	76
	HLECM (1/15)	55	44	—
	HTECM (1/6)	—	—	77
	HTECM (1/15)	59	46	—

^a Cell concentrations were the same as given in Table 1, footnote a.

^b Percentage specific ⁵¹Cr release was measured after 4.5 hr of incubation with 2×10^4 ⁵¹Cr-labeled P-815 cells/culture at Day 5 of culture.

thymocytes were injected into neonatal Balb/c hosts after a 2-hr preincubation with HTECM or control medium. The recovery after preincubation was always 85–90%. Thymocytes preincubated with HTECM showed a clear enhancement of splenomegaly, while HLECM had no effect (Table 4).

Spontaneous thymidine incorporation into spleen cells *in vitro* showed a good correlation with spleen index measurement (Fig. 3). The spontaneous thymidine incorporation into spleen cells and the spleen index of groups of mice injected with thymocytes preincubated with HTECM or HLECM are further shown in Table 5. After preincubating thymocytes with HTECM, both spleen index and spontaneous thymidine incorporation into spleen cells were enhanced.

DISCUSSION

Human thymic epithelial conditioned medium (HTECM) increases the T-mitogen response of human and mouse thymocytes (7). This effect is not seen with conditioned media from several nonthymic epithelial cultures and fibroblast cultures. This study deals with the effects of HTECM and control conditioned media on alloantigen-stimulated mouse thymocytes and on T-effector functions of mouse thymocytes. One should realize that the use of mouse thymocytes in bioassays has a number of limitations. For instance, steroid-mediated stress induces a relative enrichment of immunocompetent cells, which cells lack sensitivity as target cells in bioassays (13, 14). Also, there is a problem with respect to the viability of thymocytes in culture, which is discussed subsequently.

The data on the biological activity of HTECM obtained in our study can be compared with those of other humoral factors from the thymus, like thymosin (13, 15) and thymic humoral factor (THF) (16, 17). HTECM increases the responder capacity of mouse thymocytes in one-way MLC. This effect of HTECM is not influenced by the addition of mercaptoethanol to the culture. This distinguishes HTECM from thymosin fraction 5, which is active only in cultures without ME and with a low baseline stimulation index (13). THF has been shown to increase MLC reactivity of thymocytes only after preincubation, while the continuous presence of THF in the cultures suppressed MLC reactivity (16, 17). The addition of HTECM does not influence MLC reactivity of splenic lymphocytes at all,

TABLE 4
THE INDUCTION OF GvH REACTIVITY IN MOUSE THYMOCYTES
FOLLOWING PREINCUBATION WITH HTECM

Donor cells	No. of viable cells injected	Preincubation ^a	Spleen index \pm SE	<i>P</i>
Thymocytes	10×10^6	Medium	1.32 ± 0.12 (<i>N</i> = 17) ^b	
Thymocytes	10×10^6	HLECM	1.38 ± 0.13 (<i>N</i> = 17)	n.s.
Thymocytes	10×10^6	HTECM	1.91 ± 0.11 (<i>N</i> = 34)	<0.001
Spleen cells	5×10^6	None	2.21 ± 0.15 (<i>N</i> = 9)	

^a Thymocytes were incubated during 2 hr at 37°C with or without conditioned medium at a dilution of 1:15.

^b Number of neonates injected. In each litter one or two mice receiving 0.1 ml H-RPMI only served as controls.

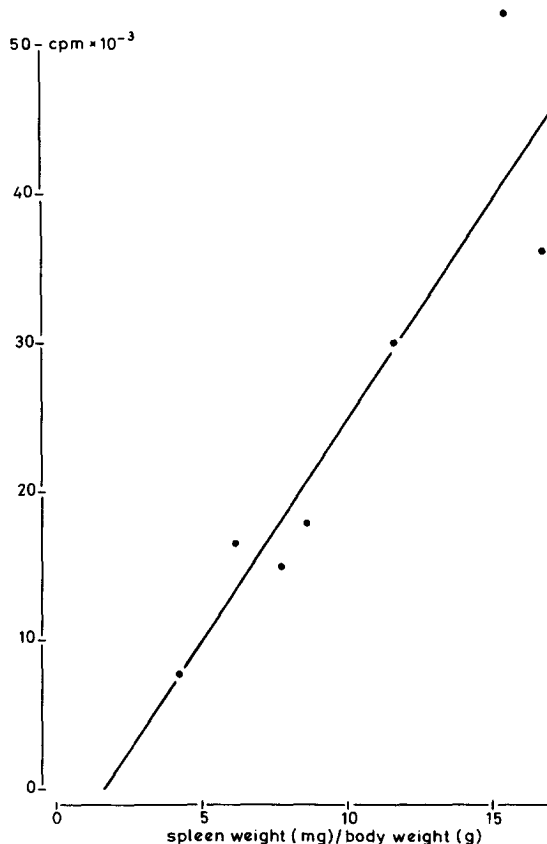


FIG. 3. Correlation between splenomegaly *in vivo* and spontaneous incorporation of thymidine into spleen cells *in vitro* ($r = 0.91$). Three different litters were injected with various thymocyte suspensions on the same day. Nine days later two parameters of GvH reactivity were determined. On the abscissa is indicated the mean ratio spleen weight (mg)/body weight (g) per group. On the ordinate is indicated the $[^3\text{H}]\text{TdR}$ uptake *in vitro* into 2×10^5 pooled spleen cells of the corresponding group/culture.

which is in accordance with data on thymic epithelial supernatant from the rat (18). Summarizing, HTECM and THF exert their biological activity under different experimental conditions, while the biological activity of HTECM and thymosin fraction 5 can better be compared since the results are obtained under similar assay conditions. Apart from this relationship in biological activity in MLC, we could also demonstrate an antigenic relationship between HTECM and thymosin by the use of antisera against thymosin fractions 5 and 6 (6, 19).

In MLC hardly any cytotoxic T lymphocytes are generated from responder thymocytes (9). HTECM can induce this generation of cytotoxic T cells in these cultures. The cytotoxic reaction of thymocytes induced by HTECM is specific for the sensitizing alloantigen, since no lysis is seen of target cells with H-2 antigens different from the stimulator cells.

The GvH reaction is often used as a test for *in vivo* immune reactivity of T lymphocytes. GvH reactivity of thymocytes is very low, i.e., high numbers of

TABLE 5
EFFECT OF HTECM ON TWO PARAMETERS OF GVH REACTIVITY OF MOUSE THYMOCYTES

	10 ⁷ thymocytes injected	Preincubation ^a	Spleen weight (mg)/ body weight (g) mean ratio \pm SD	<i>P</i>	Spleen index	Thymidine incorporation (cpm \pm SD) ^b	<i>P</i>
Expt 1	-	-	8.32 \pm 0.25 (<i>N</i> = 3)		1.0	30,500 \pm 1,011	
	+	HLECM	12.16 \pm 0.30 (<i>N</i> = 3)	<0.001 ^d	1.46 ^c	43,859 \pm 866	< 0.001
	+	HTECM	17.28 \pm 0.34 (<i>N</i> = 3)		2.07	62,781 \pm 1,112	
Expt 2	-	-	8.52 (<i>N</i> = 1)		1.0	7,917 \pm 106	
	+	HLECM	12.77 \pm 0.60 (<i>N</i> = 2)	< 0.01	1.49	21,065 \pm 607	< 0.001
	+	HTECM	17.35 \pm 0.50 (<i>N</i> = 2)		2.03	30,905 \pm 1,765	

^a Preincubation conditions were the same as for Table 4.

^b 2 \times 10⁵ pooled spleen cells/culture were assayed for spontaneous thymidine incorporation.

^c Mean spleen index obtained with thymocytes preincubated with medium was 1.32 \pm 0.12 (see Table 4).

^d HTECM versus HLECM.

thymocytes are required to induce a GvH reaction. In our experiments 10^7 Swiss thymocytes are able to elicit a positive GvH reaction. After preincubating the thymocytes with HTECM, the GvH reactivity of the cells is considerably increased. This increase was observed not only by splenomegaly measurement, but also in testing the spontaneous DNA synthesis of spleen cells *in vitro*.

Using the same batches of HTECM in all three assays, maximum activity of HTECM on mitogen responsiveness of thymocytes occurs at a dilution of 1:15 and on MLC response and cell-mediated cytotoxicity at a dilution of 1:3 to 1:6. This difference in optimal titer can be explained in various ways:

(1) The cell concentrations used in MLC and cell-mediated cytotoxicity are five times higher than in mitogen-stimulated cultures.

(2) The biological effects of HTECM are mediated by various factors. This is also suggested for several purified polypeptide components from thymosin fraction 5 (20). HTECM may also contain factors which inhibit the mitogen response and become active only at high concentrations.

(3) The difference in effects of HTECM on spleen cells suggests the existence of various target cells for HTECM. The PHA response of spleen cells is slightly increased by HTECM, but no effect is seen on the Con A response (7), MLC reactivity, and cell-mediated cytotoxicity of spleen cells.

The question arises whether the effects of HTECM on mouse thymocytes are due to the induction of unresponsive cells to responsiveness or to the selective survival or multiplication of responsive cells which are already present. In GvH experiments selective depletion of immature thymocytes seems unlikely since recovery after preincubation was always 85–90%. Further discrimination between these possibilities can be obtained from the data on cell-mediated cytotoxicity. In our experiments we measured the cytotoxic response per culture, i.e., the number of cells after culture before cytotoxicity was not adjusted. So it is very likely that the enhancing effect of HTECM on the cytotoxic activity is not based on a selective survival of cytotoxic cells, but rather on the induction or multiplication of responsive cells. With respect to the latter possibility, Hopper *et al.* (21) have reported that the absolute number of thymocytes expressing a low density of Thy-1 antigen increased by about 50% during the first day of culture. These thymocytes represent the cortisone-resistant population, in which the cytotoxic T cells are found (8, 9). However, despite this proliferation of preexistent low Thy-1 cells, no increase in progenitors of cytotoxic T cells was observed. This is in agreement with our data, in that a low cytotoxic response (less than 2%) was observed for thymocytes cultured with control media. From these data we favor the concept that HTECM is able to induce immunocompetence in immature, nonfunctional thymocytes.

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