INDUCTION OF CYTOTOXIC T CELL PRECURSORS IN VIVO

Role of T helper cells

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Under certain circumstances cytotoxic T lymphocytes (CTL)[1] are induced by processed antigen (1, 2). The mechanism of processing is still unknown, but macrophage-like accessory cells (AC) are involved (3-6). The processing presumably allows small particles of antigen to form an immunogenic association with gene products coded for by the major histocompatibility complex (MHC).

One interpretation places the requirement for processed antigen at the level of the interaction between helper T cells (T_H) and accessory cells. This interaction would generate all the soluble factors (7-10) needed for turning precursor CTL (CTLp) into effective killer cells. Since in vitro CTL can be induced by soluble factors and unprocessed antigen alone, i.e., in accessory-cell-depleted cultures (11, 12), cell-to-cell contact is deemed to be unnecessary at this step (13-15). The alternative interpretation (16) insists on the need, in vivo, for direct contact between antigen-processing cells and CTLp. The experiments to follow were designed to test these alternatives.

Materials and Methods

Rats. AS and HL rats were derived from a breeding nucleus supplied by Dr. P. Gallimore, University of Birmingham, England. From this stock, specific pathogen-free lines were developed: offspring delivered by cesarean section were foster nursed by specific pathogen-free females under barrier maintained conditions. All rats used in this study were specific pathogen free.

Tumors. The adenovirus 2 transformed lines A2/HLREF/50P/F_1/T2C4 (HL-A2T2) and A2/HLREB/10P/B1 (HL-B1), derived from HL rats, and A2/ASREB/1P/F4 (AS-F-4) derived from AS, were gifts from Dr. P. Gallimore. (17). Sp6, a spontaneous tumor isolated from the BDX strain (18), was a gift from Dr. S. Matzku, Institute for Nuclear Medicine, Heidelberg, Federal Republic of Germany.

Media. Tumor lines were maintained in RPMI 1640 supplemented with 2 mM glutamine, penicillin/streptomycin, 10 mM Hepes, and 5% heat-inactivated fetal calf serum (FCS). Media used for nylon wool purification contained 10% FCS and media for chromium release CTL assays contained 20% FCS. For the in vitro generation of CTL, Iscove's modified Dulbecco's medium (19) plus 10% FCS was used.

Preparation of Cells. Spleen and lymph node cell suspensions were prepared by dispersing the organs with a Tenbroeck tissue grinder (Technorama AG, Zurich, Switzerland). Thoracic duct lymphocytes (TDL) were prepared as previously described (20), with the exception that animals were anesthetized with a combination of Hypnorn 0.2 ml/kg (Philips-Duphar N.V., Amsterdam, The Netherlands) and Valium 2.5 mg/kg (F. Hoffmann-La Roche, Basel, Switzerland).

Nylon wool purification of TDL was based on the method of Julius et al. (21) with the modification described previously (20).

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[1] *Abbreviations used in this paper:* AC, accessory cells; Con A, concanavalin A; Con A SnF, concanavalin A supernatant factor; CTL, cytotoxic T lymphocytes; CTLp, CTL precursors; FCS, fetal calf serum; MHC, major histocompatibility complex; TDL, thoracic duct lymphocytes; T_H, T helper lymphoxytes.

Generation of CTL In Vitro. In all experiments, except those described in Table I where unmanipulated TDL were used, TDL were washed, counted, and incubated on nylon wool columns. The nonadherent population was harvested and cultured at 10^7 responder to 10^6 tumor stimulator per 20 ml of Iscove's medium or 10^7 responder to 2×10^7 lymph node stimulator cells. Tumors received 5,000 and lymph nodes 2,000 rad of x irradiation (RT 305, 300 kV, 10 mA; Phillips X-Ray, Phillips, Holland) before their use as stimulators. To some cultures 5% G-100 column purified concanavalin A supernatant factor (Con A SnF) from rat spleen cell cultures was added (22). Cultures were incubated for 6 d at 37°C in a humidified atmosphere containing 5% CO_2, in Corning 25100 tissue culture flasks (Corning Glass Works, Corning, NY).

Cytotoxicity Assay. After 6 d, which was found to be optimal, cultures were washed twice, the cells resuspended in RPMI 1640 and added, at various dilutions in 0.1 ml vol, to Greiner 96 well v-bottomed microtiter plates together with 5×10^3 ^{51}chromium-labeled tumor targets or Con A blasts. The latter were prepared by incubating lymph node cells at a final concentration of 2×10^6/ml in Iscove's medium containing 5 μg/ml Con A for 2-3 d. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 4 h, centrifuged, and 50% of the volume was harvested and counted. Values reported are the mean specific release of triplicates, provided the standard error did not exceed 10% and the specific release was >10%.

Negative Selection on Adoptive Transfer. HL/AS F_1 rats were sublethally irradiated (600 rad), cannulated within 3 d after irradiation, and injected intravenously with 3–10 $\times$ 10^9 parental (AS) TDL. Because the modal transit time from blood to lymph for T cells is shorter than for B cells (23), the population collected 6–24 h after injection is virtually all T cells (24) of donor origin. During the 6–24-h selection period all anti-host reactive T cells remain sequestered in the irradiated F_1 host lymphoid tissue (25). The selected TDL are unreactive to host alloantigens in mixed lymphocyte and local graft-vs.-host responses and normal for third-party alloantigens (26).

Negative Selection In Situ. Normal rats were injected intravenously with 20–100 $\times$ 10^6 HL-A2T2 (over this range no differences in response were observed), or 400 $\times$ 10^6 irradiated AS/HL lymph node and spleen cells. These animals were cannulated 24 h later and TDL collected for 1 d.

In some experiments tumor-injected rats were placed in a restraining cage and infused through a tail vein over a 24-h period with Con A SnF containing saline. An equivalent of roughly four spleen donors was injected per rat during this time. Control rats received Con A SnF in the absence of tumor antigen.

Results

Experimental Approach. AS and HL are two strains of rats which differ at the MHC (RT1). In the presence of Con A SnF, normal HL thoracic duct lymphocytes cultured in vitro with HL-A2T2 cells, an adenovirus-transformed line of HL origin, give high T cell-mediated (27) primary CTL responses directed to the tumor-specific antigens on HL-A2T2 cells (unpublished data). These CTL also give high lysis on other HL-derived adenovirus-transformed lines, e.g., HL-B1, but only low lysis on MHC-different AS adenovirus-transformed cells, e.g., AS-F-4 (Materials and Methods). These data suggest that the response to the tumor-specific antigens on the transformed cells is probably MHC-restricted, although definitive evidence on this point has yet to be obtained.

The experiments considered below were designed to determine whether CTLp for HL-A2T2 tumor cells exist in an MHC-different strain, i.e., in normal AS rats. Addressing this question necessitates depleting the responding AS lymphocytes of alloreactivity to HL MHC determinants. The procedure used here was to filter AS TDL from blood to lymph for 1 d through irradiated HL/AS F_1 rats (Materials and Methods). In this system donor cells with alloreactivity to the host are sequestered in the spleen and fail to enter the central lymph; recirculation of donor T cells with

other specificities is unimpeded. It should be mentioned that B cells recirculate poorly in irradiated rats, and the lymph-borne cells consist almost entirely of donor-derived T cells (24).

In addition to the above system, some of the experiments considered below employed a different system in which negative selection was induced in situ, i.e., in the donor rat itself. This procedure involves injecting rats with large doses of irradiated allogeneic cells and then collecting the host-derived cells from the central lymph 1 day later (Materials and Methods). As in the adoptive transfer selection system described above, the responding T cells become selectively sequestered in the spleen and are absent from TDL. By injecting tumor cells rather than lymphoid cells this system can be used to study negative selection of cells involved in tumor-specific CTL.

Recognition of Tumor Antigens by MHC-different T Cells. As shown in Table I, unprimed AS TDL incubated for 6 d in vitro with irradiated MHC-different HL-A2T2 tumor cells give high lysis on HL-A2T2 and HL-B1 targets and only low lysis on syngeneic AS-F-4 targets (column 1). High lysis is also observed against HL Con A blasts, which implies that some of the lysis against the HL-derived tumors is presumably directed against HL MHC alloantigens. Evidence that the HL tumors do indeed express HL MHC determinants comes from the fact that the tumors are lysed by AS TDL stimulated with normal HL lymph node cells (column 2).

Filtration of AS TDL from blood to lymph through irradiated AS/HL F_1 rats removes alloreactivity to HL. When these AS-$_{HL}$ TDL are cultured with HL-A2T2 cells, no lysis is observed on HL Con A blasts (Table I, column 3). Significantly, there is high lysis of HL-A2T2 and HL-B1 targets. This finding implies that AS TDL contain CTLp which are specific for the tumor antigens on MHC-different HL-A2T2 and HL-B1 cells.

In assessing the significance of these findings it was conceivable that the CTL activity derived from the AS-$_{HL}$-filtered T cells (Table I, column 3) reflected the presence of accessory cells. On a priori grounds these cells could have been derived either from the donor cells or from the irradiated F_1 selection host; in either situation the accessory cells would be at least semisyngeneic with respect to the responding T cells.

TABLE I

Generation of Killer Cells against HL Strain Tumors in the Absence of T Cells Specific for HL Alloantigens

Target	Column: Responder: Stimulator: E/T*:	1 AS TDL HL-A2T2 tumor 50	 25	 12	2 AS TDL HL lymph node 50	 25	 12	3 AS-$_{HL}$ TDL HL-A2T2 tumor 50	 25	 12
HL-A2T2		39	33	19	36	30	25	42	32	23
HL-B1		32	24	19	33	28	24	32	25	16
AS-F-4		12	7	4	3	1	0	‡	‡	‡
HL Con A		32	17	9	28	25	22	2	2	0
AS Con A		2	2	0	1	1	0	0	−1	0

For treatment of responders and stimulators see Materials and Methods. The data are derived from TDL from single rats. The animal in column 3 was injected with TDL from three donors.
* Effector/target cell ratio.
‡ Not tested.

TABLE II

The Requirement for Radioresistant Accessory Cells in the Generation of Anti-tumor CTL

	Column:	1			2		
	Responder:	AS nylon-wool-passed TDL			AS-HL nylon-wool-passed TDL		
	Stimulator:	HL-A2T2 tumor			HL-A2T2-tumor		
	E/T:	50	25	12	50	25	12
Culture additions							
Nil		3	2	2	−2	−1	0
AC$_{AS}$		45	44	—	46	44	30
AC$_{HL}$		28	22	—	1	2	2
Con A SnF		35	31	—	42	32	24

Normal AS and AS-HL TDL were collected as described in Materials and Methods. The nylon wool nonadherent fractions were cultured with irradiated HL-A2T2 tumor. AC$_{AS}$, AC$_{HL}$ = 2,000 rad-irradiated AS or HL lymph node cells.

To examine this question, the lymph-borne AS-HL TDL were passed through nylon wool columns and then stimulated with HL-A2T2 tumor cells, with or without exogenous accessory cells. T cells purified from normal AS TDL were used as a control. As shown in Table II, T cells fail to generate CTL activity in the absence of accessory cells. High lysis occurs, however, if the cultures are supplemented with either (*a*) Con A SnF or (*b*) syngeneic accessory cells. This applies with both T cell populations. With allogeneic (HL) accessory cells, by contrast, restoration of CTL activity only occurs with normal AS T cells and not with AS-HL T cells. These data suggest that, in the absence of alloreactivity, only syngeneic and not allogeneic accessory cells can initiate the process that leads to tumor-specific CTL.

Selective Sequestration of T$_H$ Cells and CTLp. The above findings corroborate the well-established finding that T$_H$ cells (or products in Con A SnF) control CTL generation in vitro. Depending on the conditions used, help presumably reflects interleukin production resulting from T cell contact with either (*a*) MHC alloantigens (alloreactive AS T cells confronting HL accessory cells; Table II, column 1) or (*b*) tumor antigens presented on accessory cells. In the case of CTL generated from AS-HL T cells, the simplest explanation for the requirement for syngeneic accessory cells is that help reflects MHC-restricted recognition of processed tumor antigens. Tumor-specific T$_H$ cells recognize degraded antigen on accessory cells, but only when the latter are MHC compatible.

To seek further information on the role of tumor-specific T$_H$ cells, TDL were taken from AS rats given HL-A2T2 tumor cells intravenously 1 d before. Based on the findings of other workers (28, 29), the expectation here was that accessory cells of the host would process the injected tumor cells and thereby lead to negative selection of tumor-specific T$_H$ cells. If CTLp were not selected under these conditions (the above references did not address this point directly), TDL from these rats would contain only tumor-specific CTLp and not T$_H$ cells.

The experiment shown in Table III, column 3 suggests that injecting the TDL donors 1 d previously with HL-A2T2 tumor cells does indeed remove tumor-specific T$_H$ cells but not CTLp. When TDL from these rats are filtered through AS/HL F$_1$

TABLE III

Recruitment of Tumor-specific T_H but Not CTLp after Injection of Tumor

	Column:	1			2			3		
	Responder:	AS nylon-wool-passed TDL from HL-A2T2 tumor-injected donor			AS nylon-wool-passed TDL from HL-A2T2 tumor-injected donor			AS-HL nylon-wool-passed TDL from HL-A2T2 tumor-injected donor HL-A2T2 tumor		
	Stimulator:	HL-A2T2 tumor			HL lymph node					
	Target:	HL-A2T2			HL Con A			HL-A2T2		
	E/T:	50	25	12	50	25	12	50	25	12
Culture additions										
Nil		0	0	0	15	12	5	2	0	0
AC_AS		—	2	1	22	11	3	4	2	1
AC_HL		—	41	36	—			2	0	0
Con A SnF		32	27	21	15	12	10	46	32	25

TABLE IV

T Cell Activation In Vivo to Lymphoid Alloantigens

	Column:	1			2		
	Responder:	AS nylon-wool-passed TDL from HL lymphoid cell-injected donor					
	Stimulator:	HL lymph node			HL-A2T2 tumor		
	Target:	HL Con A			HL-A2T2		
	E/T:	50	25	12	50	25	12
Culture additions							
Nil		1	1	0	1	1	0
AC_AS		0	0	1	26	22	15
AC_HL		—			1	0	2
Con A SnF		2	1	0	24	19	7

rats to remove alloreactivity and then through nylon wool columns to remove accessory cells, the T cells fail to generate anti-tumor CTL in vitro in the presence of either syngeneic or allogeneic accessory cells. By contrast, addition of Con A SnF during culture generates high CTL activity. When TDL from the tumor-injected rats are not filtered through AS/HL F_1 rats before culture, the addition of allogeneic accessory cells restores CTL activity (Table III, column 1); syngeneic accessory cells give no response. The effect observed with the allogeneic accessory cells presumably reflects a response (interleukin production) to HL alloantigens. In this respect, culturing the TDL with HL lymph node cells generates appreciable CTL activity on HL Con A blasts (column 2).

Collectively, the results in Table III suggest that preinjecting the TDL donors selectively removes only tumor-specific T_H cells. There is little or no removal of tumor-specific CTLp, even if fivefold higher doses of tumor cells are injected (data not shown). There is also no apparent removal of either T_H or CTLp reactive to HL alloantigens.

The failure to deplete alloreactivity by injecting tumor cells might be a reflection of the fact that the HL-A2T2 tumor lacks class II (Ia) molecules (30). In this respect removal of alloreactive T_H and CTLp does occur when the TDL donors are injected, not with HL-A2T2 tumor cells, but with large doses of irradiated AS/HL F_1 spleen

TABLE V

Summary of Results

| T cells tested | Negative selection of T cells reactive to: | | | |
| | HL alloantigens | | HL-A2T2 tumor antigens | |
	T_H	CTLp	T_H	CTLp
AS cells filtered through irradiated AS/HL F_1 rats	+	+	−	−
AS cells from rats injected 1 d before with AS/HL F_1 lymphoid cells	+	+	−	−
AS cells from rats injected 1 d before with HL-A2T2 tumor cells	−	−	+	−
AS cells from rats injected 1 d before with HL-A2T2 tumor cells plus Con A SnF	(−)*	+	(+)	+

Summary of data from Tables I–IV and Fig. 2.
* Inferential, not tested directly.
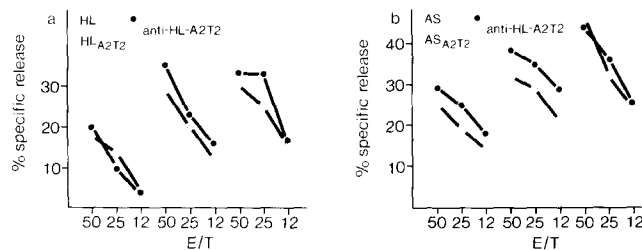
FIG. 1.   Lack of CTL recruitment to tumor antigens in syngeneic and allogeneic hosts. Three separate experiments are shown, where nylon-wool-purified TDL from normal rats or rats injected 24 h earlier with HL-A2T2 tumor are cultured with HL-A2T2 in the presence of Con A SnF. The specific chromium release in the control populations was always <7%.

plus LN cells (50% Ia$^+$) 1 d before (Table IV). When nylon-wool-passed TDL from these rats are cultured with HL lymph node cells in vitro, no CTL for HL Con A blasts are generated even in the presence of Con A SnF (column 1). The TDL thus lacked allospecific CTLp. When the TDL are cultured with HL-A2T2 tumor cells, anti-tumor CTL activity is generated, but only in the presence of syngeneic and not allogeneic accessory cells. This latter finding implies that the TDL lacked allospecific T_H cells (cells able to provide nonspecific help in response to HL alloantigens). These data are summarized in Table V.

With respect to anti-tumor CTL, it is to be noted that addition of syngeneic accessory cells does restore the capacity of TDL from F_1 spleen-injected AS rats to generate CTL to HL-A2T2 cells (Table IV, column 2). This is the same result observed in Table II, column 2, i.e., where the T cells were also depleted of alloreactivity to HL, but by in vivo filtration.

Requirements for Inducing Negative Selection of CTLp: Evidence of Two Signals.   The fact that injecting AS rats with large doses of HL-A2T2 tumor cells fails to remove either tumor-specific or allospecific CTLp is surprising, particularly since even small doses of tumor cells remove tumor-specific T_H cells. In the case of tumor-specific CTLp, it seemed possible that the MHC barrier between the injected tumor cells and the host might somehow have impeded selection. The experiment shown in Fig. 1 rules out this possibility. Here it is shown that no selection of tumor-specific CTLp occurs even
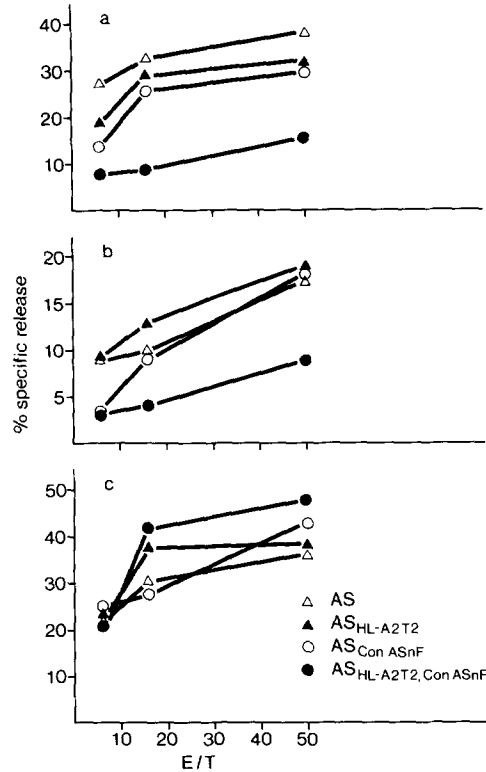
FIG. 2.   Activation of anti-tumor CTL in Con A SnF-transferred animals. Nylon-wool-purified TDL were (a) cultured and tested on HL-A2T2 tumor, (b) cultured with AC$_{BDX}$ (allogeneic and MHC incompatible to both HL and AS), and tested on Sp6, a BDX spontaneous tumor. Con A SnF was added to all flasks at the initiation of the culture period.

when HL-A2T2 tumor cells are injected into MHC-compatible HL rats.

The experiment shown in Fig. 2 was designed to examine the notion that CTLp in vivo may recognize tumor antigens (receive signal 1) but are not selected (sequestered) because they fail to receive a second signal. To test this prediction AS rats were injected with tumor cells and then perfused with Con A SnF, a presumed source of the second signal. Control rats were untreated, treated with tumor alone, or treated with Con A SnF alone. The results (Fig. 2) show a pronounced reduction in anti-HL A2T2 tumor (Fig. 2a) and anti-HL (Fig. 2b) activity in the group that received both tumor cells and Con A SnF. The control groups, given tumor or Con A SnF alone, did not differ from the untreated controls. The four groups showed equivalent reactivity against a third party alloantigen (Fig. 2c), indicating that retention of CTLp was specific for the injected antigen. A single experiment with concomitant injection of HL lymphoid and tumor cells revealed that tumor specific CTLp were not retained unless Con A SnF was also supplied.

Discussion

The results in this paper demonstrate that, in the absence of alloreactivity, activation of killer cells against allogeneic tumor antigens in vitro requires the cooperation of tumor-specific helper T cells. Stimulation of the helper cells requires

syngeneic accessory cells; these cells presumably "process" the tumor antigens and present them in association with self MHC determinants. Recognition of processed antigen is important only for T_H cells and not CTLp. Once the helper cell-accessory cell interaction has taken place, a variety of soluble factors is released and these are sufficient to induce the CTLp-CTL transition.

According to the above scheme, the activation and differentiation of CTLp requires two signals: (a) recognition of antigen on the tumor cells by CTLp and (b) a signal provided by T_H cells. Whether these signals must be received simultaneously is difficult to study in vitro since CTL are measured only after a period of 6 d. A key question is whether CTLp can recognize antigen in the absence of the second signal. A useful approach to this question is to examine the requirements for producing negative selection of T cells in vivo—a phenomenon that occurs within 1 d of contact with antigen. Since negative selection is manifested by a withdrawal of T cells from the recirculating lymphocyte pool, one might conclude that selection is simply a reflection of antigen binding by the responding T cells. If this were the case, the selection of CTLp would require only one signal. Alternatively, selection of CTLp might reflect T cell activation and thus require a second signal.

The experiments in this paper suggest that selection of CTLp does indeed require two signals. Little or no selection of either tumor-specific or allospecific CTLp occurs when the T cells are exposed to these antigens on tumor cells in vivo in the absence of Con A SnF. In the presence of Con A SnF, by contrast, effective selection occurs. In interpreting these findings two points should be emphasized. First, the precise effects of Con A SnF in promoting negative selection of CTLp are not clear. Con A SnF is known to contain a variety of lymphokines, including interleukins 1 and 2 (9) and a cytotoxic T lymphocyte differentiation factor (10). Any or all of these factors might control negative selection. Secondly, in contrast to tumor cells, injection of allogeneic lymphoid cells does select allospecific CTLp in the absence of Con A SnF (Table IV). Although this requirement for Ia^+ cells as the targets for negative selection has yet to be proved, one could envisage that these cells simultaneously attract both T_H cells and CTLp, with the result that the CTLp are exposed to a high local concentration of helper factors (signal 2). Alternatively, the Ia^+ cells themselves might release factors which facilitate recognition of antigen by the CTLp.

The model of CTL activation developed in this study fits also several observations that were difficult to reconcile with data provided by in vitro experiments. Zinkernagel et al. (31) and von Boehmer and Haas (32) demonstrated a requirement for I region sharing in the generation of CTL in vivo. Keene and Forman (33) showed that for effective priming to occur in vivo helper determinants required for expression of CTL activity must be on the same cell as the CTL determinants. Korngold and Sprent (34) reported that the induction of graft-vs.-host disease requires contact with antigen on marrow-derived (AC?) cells, whereas the effector cells were targeted to Ia negative non–marrow-derived cells. In each case the immunogenicity of a target antigen for a K,D-restricted CTL would appear to controlled by the I region. These data are in apparent disagreement with a study of Doherty and Bennink (35), who concluded that virus-specific CTL can be activated to antigen in Ia-incompatible irradiated mice. These authors used an in vivo filtration system to remove alloreactive T cells but did not attempt to remove accessory cells from the filtered T cell population. Hence carry-over of accessory cells might explain their results (Table II and reference

36).

Finally, comment should be made on the following paradox. It was mentioned earlier (Results) that primary responses to syngeneic adenovirus-transformed tumors are low unless the cultures are supplemented with Con A SnF. This finding implies that unprimed rats are relatively deficient in tumor-specific T_H cells. In allogeneic situations, by contrast, high primary antitumor responses are observed in the absence of Con A SnF (Table II); the response occurs in the apparent absence of alloreactive T cells and requires syngeneic accessory cells. In the light of this finding, one reaches the surprising conclusion that the precursor frequency of tumor-specific T_H cells is much higher when the antigens are derived from MHC-incompatible tumor cells. Possible explanations for this unexpected finding are discussed elsewhere.[2]

Summary

Strain AS rats respond with two populations of cytotoxic T lymphocytes to stimulation in vitro by the major histocompatibility complex (MHC)-incompatible strain HL rat tumor (HL-A2T2). One is specific for MHC alloantigens present on both HL-A2T2 and normal HL targets, the other is tumor specific. The activation of these killer cells requires helper T lymphocytes. The tumor-specific helper cells depend on syngeneic radioresistant accessory cells to present the tumor antigens in an immunogenic form. The appropriate helper-accessory cell interaction results in the production of soluble factors which then induce the maturation of precursor cells into effective killer cells.

Studies with a procedure for inducing negative selection of T cells in vivo showed that short-term exposure to HL-A2T2 tumor induced selection only of T_H but not cytotoxic T lymphocyte precursors (CTLp). Simultaneous injection of supernatants from concanavalin A-activated spleen cell cultures, however, did produce selection of CTLp. These and other findings suggest that under normal circumstances in vivo, both signals (recognition of antigen and acceptance of maturation factors) are provided in the vicinity of an antigen presenting macrophage-like accessory cell.

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