Elicitation of Anti-Leukemia Cytotoxic Responses*

Bach, F. H., Sondel, P. M., Zarling, J. M.

Immunology Research Center and the Departments of Medical Genetics. Surgery. Pediatrics and Human Oncology. University of Wisconsin. Madison. Wisconsin 53706. USA

A major goal in our understanding of leukemia involves the study of cell surface antigens on the leukemic cells that are not present on patients' normal cells of the same type. These antigens, if they serve as tumor rejection type antigens, are commonly referred to as tumor associated transplantation antigens. One approach to the study of these antigens, which is also of potential interest with regard to cellular immunotherapy of leukemia, involves attempts to generate cytotoxic cells against autologous leukemia cells. The approaches that we have used have largely been based on earlier and concurrent studies involving recognition and response to alloantigens in the mixed leukocyte culture (MLC) and cell mediated lympholysis (CML) assays. Two general approaches have been used, both of which are presented in this paper. In each case the background studies of the allogeneic system are given as a reference.

Methods Employed

The mixed leukocyte culture (MLC) test [1,2] is most commonly assayed by studying the incorporation of radioactive thymidine into the dividing cells in the responding cell population. Stimulating cells are treated with x-irradiation or mitomycin-C [3] so that they will present their foreign antigens to the responding cells but will not themselves respond, i.e. incorporate radioactive thymidine. It is now recognized that both helper T lymphocytes (T_h) and cytotoxic T lymphocytes (T_c) respond proliferatively in a mixed leukocyte culture; in addition, in all probability suppressor T lymphocytes (T_s) also respond. In fact, it is not critically established whether the T_h cells do themselves proliferate in a mixed leukocyte culture. However, proliferation of the responding cells has most commonly been associated with a helper response in that the requirement for T_h activity, in terms of the development of cytotoxic cells, is present. Whereas in a primary mixed leukocyte culture, more than 90% of the dividing cells belong to the T lymphocyte subclass cells that

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carry the Ly 1 differentiation antigen [4,5] (which we associate with helper T lymphocyte activity), it would probably be dangerous to equate proliferation to T mediated "help" under all MLC-like conditions.

Cytotoxic T lymphocytes (T_c) are generated in mixed leukocyte cultures and can be measured in the cell mediated lympholysis assay [6–8]. The T_c cells are tested for their ability to lyse radioactive sodium chromate (^{51}Cr) labeled target cells that carry the sensitizing antigens.

In addition to the generation of T_h and T_c cells in an MLC, suppressor T lymphocytes are also generated. These cells are usually detected by their ability to suppress autologous responding cells from differentiating into T_c in response to unrelated alloantigens in a fresh MLC.

The Three-Cell Approach to Generating Killer T_c Cells

In vitro T lymphocyte reactivity to allogeneic cells in man appears to be restricted almost entirely, in a primary sensitization system, to a response against antigens of the major histocompatibility complex, HLA. Within the HLA complex, as with the mouse homolog of that complex (H-2), there are two types of antigens which are commonly referred to as LD (lymphocyte or MLC defined) and CD (cytotoxicity or CML defined) antigens insofar as these antigens are recognized by the T lymphocytes. The LD antigens in man are associated with the HLA-D locus and in mouse with the H-2 I region; the CD determinants are associated with the HLA-A, -B and -C loci in man and primarily with the H-2 I region in mouse [9]. The finding of greatest note with regard to the principle emerging from studies involving allogeneic cells as it relates to the generation of cytotoxic cells against autologous leukemia cells is the following. If responding T lymphocytes of one individual are stimulated with cells that differ by CD determinants but do not differ by LD antigens, then the cytotoxic response which develops against the CD determinants is markedly weaker than when the stimulating cells include both an LD and a CD stimulus [9].

These findings are illustrated in a schematic fashion in Table 1. The ap-

Table 1. A three-cell experiment for the generation of cytotoxic T lymphocytes

Sensitizing MLC	HLA difference between responding and stimulating cell	^{3}H-TdR incorporated	% CML target cell		
			A	B	C
AB_m	HLA-D	+ + + +	–	–	–
AC_m	HLA-A, -B and -C	–	–	–	–
AB_mC_m	HLA-A, -B, -C and -D	+ + + +	–	–	+ + +

Typical results of CML studies in a family in which a recombinational event has taken place. Siblings A and B are identical for the HLA-A, -B, and -C loci but differ for HLA-D. Siblings A and C differ for the three SD loci, HLA-A, -B, and -C but are identical for HLA-D. In the three-cell experiment, A is simultaneously stimulated with the cells of sibling B and the cells of sibling C. [Reprinted from Bach, F.H.: Ann. Rev. Genet. **10**, 319–339 (1976)].

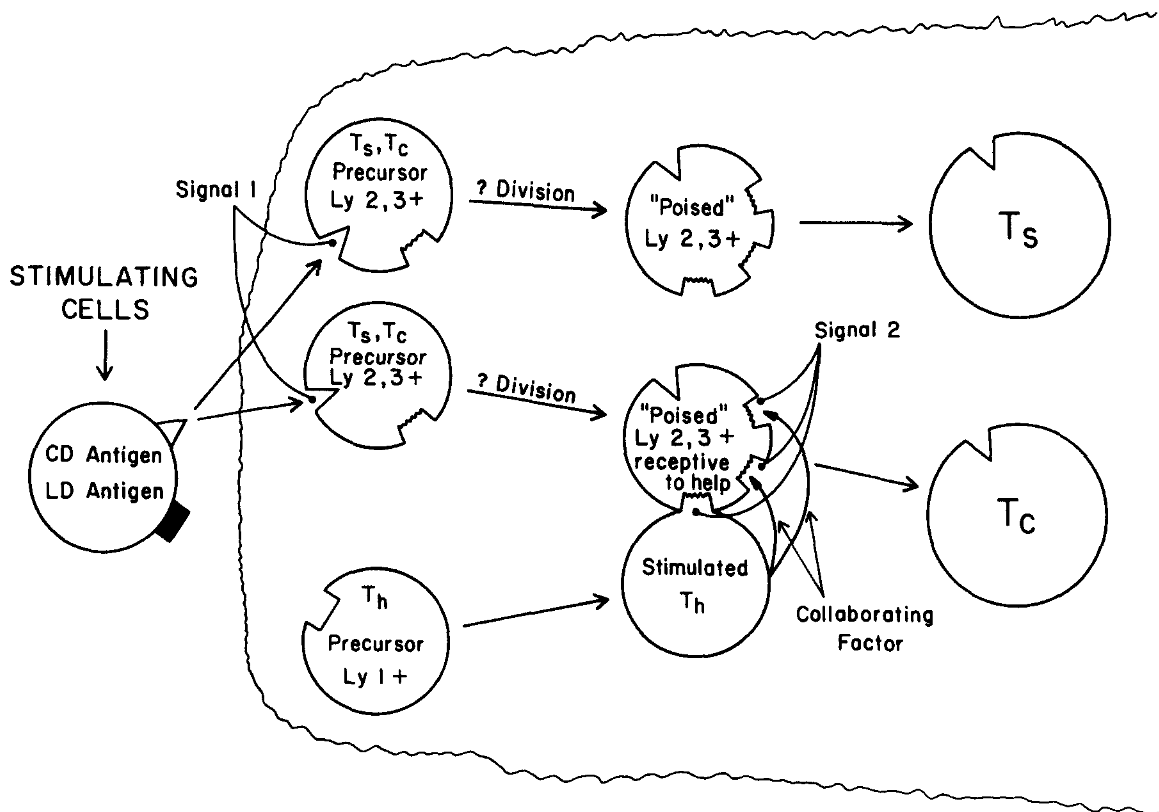

Fig. 1. A model illustrating the signals involved in the development of T_c in a primary response. This represents a modification of the model presented previously [9]

parent basis for this phenomenon, which we have referred to as LD-CD collaboration, is that functionally different populations of T lymphocytes respond preferentially to LD and CD determinants. It is the T_h cells that primarily recognize LD antigens and the T_c cells that recognize the CD determinants. In a manner that is not clearly understood at the present time, the reaction of T_h cells to the foreign LD determinants on the allogeneic cell provides "help" to the precursor cytotoxic T lymphocytes that have recognized the allogeneic CD determinants. This results in the development of a stronger cytotoxic response. The cellular model, for which extensive experimental support exists, depicting response to LD and CD determinants is shown in Fig. 1.

The greater cytotoxic response engendered when both foreign LD and CD determinants are present as stimulating determinants can be obtained by using stimulating cells from a single individual who differs from the responding individual by both LD and CD determinants. Alternatively we can use the "three-cell protocol" wherein the responding lymphocytes are simultaneously cultured with stimulating cells from one individual who differs by CD determinants and with stimulating cells from a second individual who differs by LD determinants [9–11]. The precursor T_c cells of the responding cell population presumably recognize the CD determinants on the stimulating cells of one individual (depicted as C in Table 1) whereas the T_h cells respond to the LD determinants on the cells of a second individual (depicted as in-

dividual B in Table 1). It is the interaction between the T_h and T_c cells in this "three-cell protocol" which leads to the amplified cytotoxic response. In some situations, if the stimulating cells are presented in such a way that they present their CD determinants without "any" LD stimulus, no cytotoxic response is generated. However, if third party LD different, helper-stimulating cells are added, a cytotoxic response is developed against the stimulating cells that differ from the responder with regard to CD, but not LD, determinants. This last observation is particularly important for our interpretation of the data with leukemia.

Application of Three-Cell Protocol to Leukemia

Leukemic blast cells from an individual who has active disease at the time of taking the cells do not, as a general rule, stimulate a cytotoxic response in the remission, normal lymphocytes of that same individual. This is illustrated in Table 2 [12]. On the assumption that the leukemic blast cells may in fact express a CD-like target determinant which the responding autologous cells can "see" but to which they cannot respond because there is no helper stimulus available, we have used the "three-cell" approach. We have added stimulating cells of an unrelated individual who differs from the patient with leukemia by HLA-D determinants (i.e. LD antigens) to a mixture of responding lymphocytes and autologous leukemia stimulating cells. Under these conditions, we

Table 2. In vitro generation of lymphocytes cytotoxic for autologous acute myelomonocytic leukemia cells by a "three-cell" protocol

Responding lymphocytes	Stimulating cells	Effector/target cell ratio	% Specific ^{51}Cr release ± S.D.			
			Leukemic cells	Remission marrow cells	Normal lymphocytes from A	Normal lymphocytes from B
C (patient's lymphocytes)	L_x (patient's leukemia cells) + A_x (normal allogeneic lymphocytes)	40:1	17,8 ± 6,5	3,5 ± 1,4	44,0 ± 4,1	1,8 ± 2,3
C	L_x	40:1	0,0	0,0		
C	A_x	40:1	2,9 ± 1,1	0,45 ± 1,6	60,7 ± 10,1	2,2 ± 2,2
B (normal individual)	L_x	20:1	17,5 ± 12,0	14,7 ± 2,0	–0,7 ± 3,4	–0,4 ± 2,8
B	A_x	20:1			27,7 ± 2,3	–0,2 ± 3,4

Effector cells were collected 7 days after stimulation, and incubated with 1×10^4 ^{51}Cr-labelled target cells at the designated effector: target cell ratios. The % specific ^{51}Cr release was measured after a 6 hour incubation of effector cells and target cells at 37°C. [Reprinted from Zarling et al.: Nature **262**, 691–693 (1976)]

Table 3. Generation of cytotoxic lymphocytes against an HLA identical sibling's acute lymphocytic leukemia (ALL) cells by a "three-cell" protocol[a]

Responding cells	Stimulating cells	% Specific ^{51}Cr Release ± S.D.[b] Target cells	
		Sib 2 lymphocytes	Patient's ALL blasts
Sib 1	Media	− 16.0 ± 5.7	9.8 ± 2.0
Sib 1	Sib 2_m	− 9.4 ± 5.7	6.8 ± 1.5
Sib 1	Z_m	− 6.5 ± 5.7	21.4 ± 2.0
Sib 1	Pt. ALL $blasts_m$	− 10.6 ± 5.7	8.6 ± 1.8
Sib 1	Pt. ALL $blasts_m$ + Z_m	− 7.2 ± 5.9	38.7 ± 1.9
Z	Pt. ALL $blasts_m$	46.6 ± 8.9	46.2 ± 3.0

[a] The ALL patient whose blasts are studied here (Pt. ALL blasts), and his siblings, Sib 1 and Sib 2, were found identical for HLA-A and HLA-B haplotypes following serological typing of all siblings at both parents. Individual Z is unrelated..

[b] Six days following mixed leukocyte culture of Sib 1's or 2's lymphocytes with mitomycin-treated$_{(m)}$ stimulating cells, the cell mediated lysis experiment was performed using a ratio of 20 effector cells: ^{51}Cr labeled target cell. Sensitization of Sib 1's cells with mitomycin-treated HLA identical ALL blasts together with mitomycin-treated allogeneic cells (Pt. ALL $blasts_m$ + Z_m) resulted in the generation of a strong cytotoxic response against the patient's blasts but not against the other sibling's (Sib 2) lymphocytes. [Reprinted from Sondel et al.: J. Immunol. **117**, 2197–2203 (1976)].

have found that in a certain number of cases, we have been able to generate cytotoxic T lymphocytes that are capable of lysing the autologous leukemia cells but do not lyse normal target cells of the same individual.

An alternative approach to this problem has been to use an HLA identical sibling as the responding cell donor and ask whether these cells can generate a cytotoxic response against the leukemic cells of the HLA identical sib. (Lymphocytes of normal HLA identical siblings do not stimulate each other to produce a cytotoxic response.) As with the autologous situation, in most instances in which this protocol is attempted, when just the leukemic blast cells are used as the stimulating cells, no cytotoxic response is generated. On the other hand as depicted in Table 3 [13], a cytotoxic response is generated in some cases when the three cell approach is utilized. These findings using the three-cell approach have received extensive confirmation from the recent studies of Lee and Oliver [14].

Pool Sensitization for Generating T_c Cells

Several years ago, we noted that if cells from 20 unrelated individuals, chosen at random, are mixed in a "pool" in equal numbers, then the resulting "pooled stimulating cell" would cause a very strong proliferative response in lymphocytes from any one individual, the response being at least as strong and in most cases stronger than that induced by any one allogeneic stimulating cell [15]. Subsequent studies by Sondel et al. [16] and Martinis and Bach [17] demonstrated that stimulation with the pooled stimulating cells apparently resulted

Table 4. Lysis of autologous hairy cell leukemia cells by T cells sensitized to pooled allogeneic normal cells[a]

Responding cells	Stimulating cells	% ^{51}Cr Release ± S.D. Target cells			
		Patient 1's splenic leukemic cells	Patient 1's peripheral leukemic cells	Patient 1's peripheral T cells	Individual A's peripheral T cells
Exp. a					
P1 (patient 1)	P1-L_x (patient 1's leukemia cells)	1,7 ± 2,3		0,5 ± 3,0	
P1	$pool_x$	24,5 ± 1,8		0,8 ± 2,5	
Exp. b					
P1 (patient 1)	P1-L_x (patient 1's leukemic cells)	–1,0 ± 0,5			
P1	A_x	5,3 ± 2,6			37,7 ± 3,9
P1	$pool_x$	19,0 ± 3,7	21,1 ± 0,8	–1,5 ± 2,7	36,4 ± 5,6
A (norm. individ.)	P1-L_x	32,8 ± 2,6			
A	$pool_x$	29,5 ± 2,8	45,5 ± 3,1	15,8 ± 2,7	–9,2 ± 3,4
		Patient 2's splenic leukemic cells	Patient 2's peripheral leukemic cells	Patient 2's peripheral T cells	Individual A's peripheral T cells
Exp. c					
P2 (patient 2)	P2-L_x (patient 2's leukemic cells)	–2,6 ± 1,7			
P2	$pool_x$	18,1 ± 5,6	27,2 ± 3,6		
A (norm. individ.)	P2-L_x	25,2 ± 4,1	21,0 ± 2,6	16,8 ± 4,9	–3,6 ± 2,0
Exp. d					
P2 (patient 2)	$pool_x$	18,9 ± 1,5		–0,3 ± 2,5	
B (norm. individ.)	$pool_x$	32,7 ± 2,9		41,0 ± 5,4	

[a] T cells from normal individuals A and B and from the patients with hairy cell leukemia (P1 and P2) were isolated as described in the methods and the T cells were stimulated with the leukemia cells (P1-L_x and P2-L_x), with cells from a single normal individual (A_x) or with normal cells pooled from 20 individuals ($pool_x$). The ratio of stimulating cells to responding T cells was 1 : 1 in this experiment, however, in no case when the patients' T cells were stimulated with autologous leukemia cells at ratios of 1 : 1 to 2 : 1 was cytotoxicity against the leukemia cells detected (data not shown). T cells from the normal individuals and the patients, that were cultured in the absence of stimulating cells, were not cytotoxic for the leukemic cells (–2,3 ± 1,4 to 0,6 ± 2,0% ^{51}Cr release). [Reprinted from Zarling et al.: Nature 274, 269–271 (1978)].

in activation of essentially all cells that recognize LD determinants as well as all cells that recognize CD determinants that differ from those expressed on the responding cells. The latter finding was based on the demonstration that the cytotoxic T lymphocytes that are generated by stimulation with the pool killed virtually any allogeneic cell. Subsequent studies in our laboratory demonstrated that sensitization with the pool also led to the development of cytotoxic cells capable of killing autologous lymphoblastoid cell lines derived by Epstein-Barr virus transformation [18].

We have studied the ability of pool sensitized cells to lyse autologous leukemia cells in two patients with hairy cell leukemia. Results from those studies, shown in Table 4 [19], demonstrate that in these two cases of leukemia the pool sensitized cells are able to cause significant lysis of the leukemia cells but not autologous normal lymphocytes.

The reasons why pool sensitization is an effective method of generating T_c cells against autologous lymphoblastoid cell line or leukemia targets in at least some cases are not understood. Several possibilities exist. First, it may be that the CD determinants present on the allogeneic cells cross-react with the "CD-like" determinants recognized as foreign on the autologous abnormal cells. Alternatively it may be that pool sensitization leads to some form of "polyclonal" activation in that all clones of lymphocytes of different functional subcategories are activated by pool stimulation; there is no evidence to support such a suggestion for pool stimulation. Whether the target antigens recognized after pool-sensitization are tumor-associated antigens associated with the leukemogenic process, normal differentiation antigens expressed on the leukemic cells as they are on the precursors from which leukemia was derived, or are derepressed normal histocompatibility antigens is not clear.

Summary

We have presented the rationale for the in vitro approaches that we have taken for generating cytotoxic lymphocytes capable of lysing autologous leukemia cells or leukemia cells from HLA identical siblings. Two different approaches have been used, both of which are based on earlier findings concerning the antigenic and cellular interactions involved in the generation of strong cytotoxic responses to alloantigens in mixed leukocyte culture.

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