SECONDARY CELL-MEDIATED LYMPHOLYSIS: IMPORTANCE OF *H-2* LD AND SD FACTORS*‡

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Andersson's and Häyry's description in 1973 (1) of a secondary-type response in vitro after "sensitization" of lymphocytes in mixed leukocyte cultures (MLC)[1] opened an area of immense interest. The basic observation now studied in several laboratories (2–7) was that after a period of in vitro incubation in MLC, at a time when the proliferative and cell-mediated lympholysis (CML) responses have either returned to background levels or at least diminished markedly from the peak reactions, restimulation of the cells remaining in culture with lymphocytes of the original sensitizing cell donor results in rapid and strong development of proliferative and cytotoxic responses.

In a primary MLC-CML reaction, it appears that major histocompatibility complex (MHC) LD antigens are for the most part responsible for the stimulation of proliferative events in a subpopulation of responding T cells, the proliferating helper cells (PHCs). The MHC SD antigens, or products of loci very closely linked to those determining the SD antigens, are primarily responsible for the activation of, and serve as targets for, a second lymphocyte subpopulation, cytotoxic T cells (CTLs) in the CML phase. We have recently obtained preliminary data in man (4) which suggest that after sensitization of a primary MLC with both MHC SD and LD differences, restimulation of the secondary culture with an LD difference will result in not only a secondary proliferative response but a significant and rapid redevelopment of cytotoxicity against the SD antigens present in the primary sensitization (5). In the present report we extend and more critically analyze these findings in mice and present results of experiments designed to elucidate the role of MHC LD and SD components in the secondary stimulation system.

Materials and Methods

Mice. Inbred strains used in this study and maintained in this laboratory are B10.A *(kkdd)*, B10.T(6R) *(qqqd)*, AQR *(qkdd)*, C57BL/10 *(bbbb)*, B10.S *(ssss)*, B10.D2 *(dddd)*, B10.G *(qqqq)*, and

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[1] *Abbreviations used in this paper:* CML, cell-mediated lympholysis; CTLs, cytotoxic T cells; FCS, fetal calf serum, MHC, major histocompatibility complex; MLC, mixed leukocyte cultures; MR, maximum release; PHCs, proliferating helper cells; SR, spontaneous release.

B10.BR *(kkkk)*. Lowercase italic letters in parentheses after the strains refer to the *K, I, S,* and *D* regions of the MHC and represent their *H-2* designation.

We refer to two mouse strains as "LD different" if, with respect to *H-2*, they differ for the strong *LD* locus in the *I* region but are identical for *H-2K* and *H-2D*. Two mice are called SD different if they differ for the *K* and/or *D* region and are *I*-region identical. The designation SD and LD are simply terms we use to allow one to differentiate between MHC determinants that may have different biological roles. The terms should not imply that a function associated with LD cannot also be associated with SD; for instance, that SD antigens cannot induce lymphocyte proliferation.

Primary Sensitization. Mouse spleen cell allosensitization in MLC has been described elsewhere (8). Briefly, 50×10^6 responding spleen cells are cultured with 50×10^6 mitomycin C-treated stimulating spleen cells in 20 ml of EHAA media containing 2-mercaptoethanol (5×10^{-5} M) and 0.5% mouse serum (from the responder strain) in upright no. 3013 Falcon tissue culture flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in 5% CO_2 in air (humidified) (8). After 5 days in culture, the proliferative MLC response is measured by the incorporation of [^{3}H]thymidine (2 μCi/culture) into triplicate 200-μl samples (multiple automated sample harvestor; Otto Hiller Co., Madison, Wis.) of reacting cells and expressed as the mean counts per minute ± standard deviation (SD). The primary CML response is also determined on day 5.

Secondary Sensitization. Spleen cell cultures prepared as for primary sensitization are incubated for 13–17 days at which time they are assayed to determine the remaining MLC (proliferative) and CML activity. The primed cells (2×10^5 cells per well) are then incubated with fresh restimulating (mitomycin C-treated) spleen cells (2×10^5 cells per well) from various strains of mice in Linbro (IS-MRC-96-TC) round-bottom microtiter plates (Linbro Chemical Co., New Haven, Conn.). Proliferative and cytotoxic responses are then measured on days 1–5 after secondary stimulation.

CML. The CML procedure has been described elsewhere (8). Briefly, effector cells obtained from MLC are resuspended in EHAA plus 5% inactivated (56°C, 2 h) fetal calf serum (FCS) at the viable cell concentration to be used in the CML. Target cells are lymph node cells that have been stimulated with phytohemagglutinin-M for 48 h, labeled with $Na_2{}^{51}CrO_4$, washed in Hanks' balanced salt solution, and resuspended in EHAA + 5% FCS (inactivated) at a viable cell concentration to yield 1×10^4 cells per effector-target mixture. Target and effector cells (100-μl portions of each) are incubated in Linbro round-bottom microtiter plates (no. IS-MRC-96; Linbro Chemical Co.) for 3–3.5 h after which the plates are centrifuged and ^{51}Cr released in the supernate determined. Spontaneous release (SR) represents the counts per minute of the supernate from 1×10^4 target cells alone. Maximum release (MR) is determined by detergent lysis of 1×10^4 target cells. Percent CML is calculated as follows:

$$\frac{(\text{cpm experimental release} - \text{cpm SR})}{(\text{cpm MR} - \text{cpm SR})} \times 100.$$

Results

Fig. 1 shows results of a typical restimulation experiment where primary sensitization to LD and SD differences is followed by secondary stimulation by these same antigenic differences. The proliferative and cytotoxic responses at various times after initiation of the primary and secondary cultures are given. By the time of restimulation the proliferative response of the primary MLC has diminished and the primary CML response, while usually still significant, is also markedly reduced as compared with its peak response on day 5. Despite the remaining CML on day 14, a significant increase takes place within only 24 h after restimulating cells syngeneic to the primary stimulator are added; the maximum cytotoxic response usually occurs between day 2 and day 4. Addition of restimulating cells syngeneic with the responding cells has little effect on either the proliferative or the cytotoxic responses.

Role of LD in Restimulation. We first tested the ability of LD differences alone to restimulate a culture whose primary sensitization was against both LD

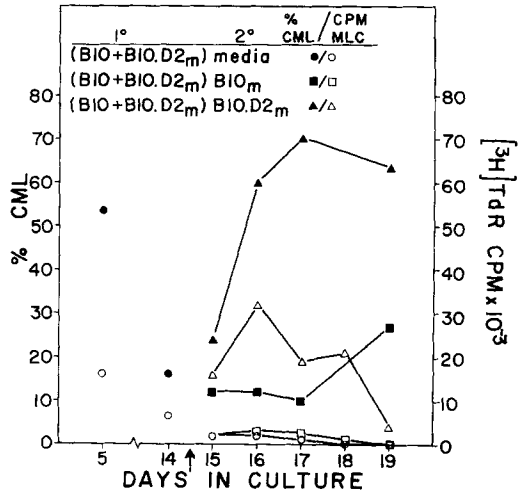

FIG. 1. Primary MLC and CML on B10.D2 target cells and kinetics of secondary MLC and CML on B10.D2 target cells. B10 spleen cells sensitized to B10.D2 mitomycin C-treated spleen cells are assayed for primary MLC (open symbols) and CML (closed symbols) on day 5 and day 14 of culture. On day 14, primed cells are restimulated (↑) with media only (●, ○). $B10_m$ spleen cells (■, □), or $B10.D2_m$ spleen cells (▲, △). CML, expressed as percent CML; and MLC, as counts per minute of incorporated [^{3}H]TdR, are determined 1, 2, 3, 4, and 5 days after secondary stimulation. All points shown for cytotoxicity are effector:target ratios of 30:1 and are either actual values or values extrapolated from effector to target ratio from dose response curves of at least three point determinations.

and SD differences. B10.T(6R) *(qqqd)* cells are sensitized in primary culture to B10.A *(kkdd)* (LD and SD differences) and restimulated in the secondary phase with AQR *(qkdd)* (LD difference only). Results are shown in Fig. 2. After sensitization in the primary there is high level CML on B10.A with low level CML on B10.S and AQR. On day 16, 2 days after restimulation, excellent and rapid CML has developed against the B10.A target regardless of whether restimulation is done with B10.A, AQR, or B10.S cells. Each of these cells also induces a "secondary-type" proliferative response, i.e., a significant response by day 2 after restimulation (data not shown). Restimulation with AQR or B10.S in this experiment does not result in significant levels of CML directed at these targets within the first 2 days after restimulation.

In view of our recent findings that LD differences alone are sufficient to generate relatively weak, but significant, CML in primary culture (8, 9), we have tested their ability to restimulate a secondary anti-"LD" CML response. As illustrated in Table I, using the LD disparate strains 6R *(qqqd)* and AQR *(qkdd)* for primary sensitization, secondary stimulation on day 14 with the original LD-different sensitizing strain (AQR) leads to a rapid proliferative response, which is comparable to that seen when both LD and SD differences are present in the two phases of the reaction (data not shown). Furthermore, 2 days after secondary stimulation, the relatively low level cytotoxicity directed at the LD-different sensitizing cell in the primary CML (day 5), is enhanced to levels equal to or greater than those seen in the primary (Table I). When cells from strain B10.A *(kkdd)* are used to restimulate the 6R plus AQR_m sensitized culture, similar

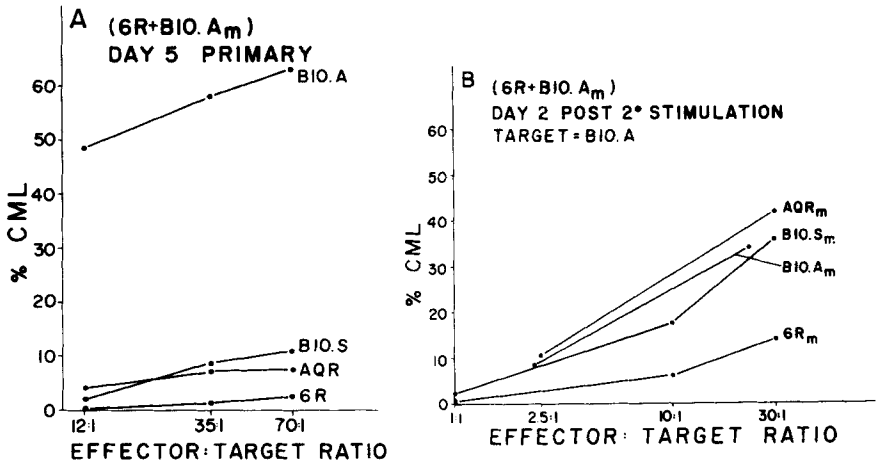

FIG. 2. Cytotoxic activity of primary culture sensitized to LD and SD antigens and restimulated with LD antigens alone or LD plus SD antigens. Part A shows the cytotoxic response of 6R spleen cells sensitized to B10.A mitomycin C-treated spleen cells on day 5 of a primary response on target cells of B10.A (specific sensitizing strain, LD plus SD different from 6R), AQR (LD identical to B10.A and LD different from 6R), B10.S (LD and SD different), and 6R (control). On day 14, immediately before and the day of restimulation, the 6R plus B10.A_m sensitized cells gave the following CML response at 75:1 effector:target ratio on targets:B10.A, 22.5%; 6R, −4.8%; AQR, −2.4%; and B10.S, −2.7%. Part B shows the CML response on B10.A target cells 2 days after restimulation of the 6R plus B10.A_m culture with AQR_m, B10.S_m, B10.A_m, or $6R_m$. Cytotoxicity on targets AQR, B10.S, and 6R ranged from −8.1 to 4.1%.

secondary-type proliferative and cytotoxic responses result. This is as expected since B10.A and AQR share the *I* region and they presumably share the same LD. The question, however, remained whether after sensitization to the primary LD stimulus, the SD antigens of B10.A which differ from the responding 6R cells would generate an additional cytotoxic response in the secondary. The results given in Table I are consistent with the concept that although the SD antigens of B10.A apparently do not generate additional secondary-type cytotoxic cells against themselves, at least at this early time of assay after restimulation, the LD differences of B10.A can substitute for those of AQR in restimulation of the anti-LD CML.

Role of SD Antigens in Restimulation. After primary stimulation with a combined LD and SD difference, target cells carrying the SD antigens of the stimulating cell are lysed extensively in the CML assay. Target cells carrying SD antigens which are presumably cross-reactive with those of the sensitizing cell (10) can be lysed to a lesser, but significant, extent. These same cross-reacting strains can, if used as donors of restimulating cells, lead to the rapid development of CML against target cells from the original sensitizing strain, presumably at least in part, via LD stimulation of the culture. The experiments to be described below were done to test whether the SD antigens present on the restimulating cells in some way affect the magnitude or specificity of the CML response directed at the various targets.

TABLE I

CML after Primary LD Sensitization and Secondary LD or LD plus SD Restimulation

MLC (mean cpm ± SD)	Stimulus	Day of assay	Effector: target ratio	% CML ± SD of targets		
				B10.T (6R) (*qqqd*)*	AQR (*qkdd*)	B10.A (*kkdd*)
	Primary					
30,862 ± 2,428	6R+ AQR_m	5	75:1	4.8 ± 2.1	19.5 ± 4.8	14.9 ± 2.2
–		14	30:1	2.2 ± 1.5	−0.3 ± 2.1	–
	Secondary					
4,841 ± 1,367	+ $6R_m$	2‡	30:1	−7.5 ± 3.3	5.2 ± 7.6	–
62,133 ± 5,915	+ AQR_m	2	30:1	–	33.1 ± 6.2	26.2 ± 1.7
62,524 ± 3,093	+ $B10.A_m$	2	30:1	–	24.6 ± 6.9	31.7 ± 2.6

* Lowercase italic letters refer to the regions of the MHC as described in the text.

‡ The day of assay of secondary cultures refers to the number of days after secondary stimulation (on day 14) when the CML and MLC responses are determined.

Cells of B10 *(bbbb)* were sensitized in a primary MLC with cells of strain B10.D2 *(dddd)*. These cultures were restimulated on day 14 with B10.D2, B10.G *(qqqq)*, or B10.BR *(kkkk)*. CML was tested on target cells of each strain. Results from one such experiment are given in Fig. 3. On day 5 after primary sensitization with B10.D2, there is approximately equal cross-killing on B10.BR and B10.G targets. The addition of any of these cells as restimuli results in strong killing against the original sensitizing cell, B10.D2 on day 16, 2 days after secondary stimulation on day 14. In addition, these experiments demonstrate that the SD antigens on either the B10.BR or the B10.G-restimulating cell will "deviate" the response toward that particular cell. Note, for instance, that after primary sensitization to B10.D2 and restimulation with B10.D2, there is slightly stronger cross-killing on B10.G than on B10.BR. Yet, if B10.BR is used as the restimulating cell, the CML on B10.BR is now greater than on B10.G; if B10.G is used as the restimulating cell, the CML on B10.G is markedly greater than on B10.BR. Comparable results (not shown) have been obtained using B10.G as the primary sensitizing cell. In every case the level of CML in the secondary culture is maximal against the target syngeneic to the original sensitizing cell. It is important to emphasize that the deviation of the respone noted above, presumably by the SD antigens on the restimulating cells, was observed in those cases where the restimulating cells were thought to have SD antigens cross-reactive with the SD antigens of the original sensitizing cells, as indicated by cross-killing in the primary CML assay.

Discussion

The genetic and cellular dichotomy of MHC antigens that play a role in the generation of a primary MLC-CML reaction has been intensively studied in the last few years (11–15). The finding that in primary MLC-CML the T-cell subpopulation that mediates cytotoxicity is physically separable from the T-cell subpopulation that is primarily responsible for the proliferative response in MLC

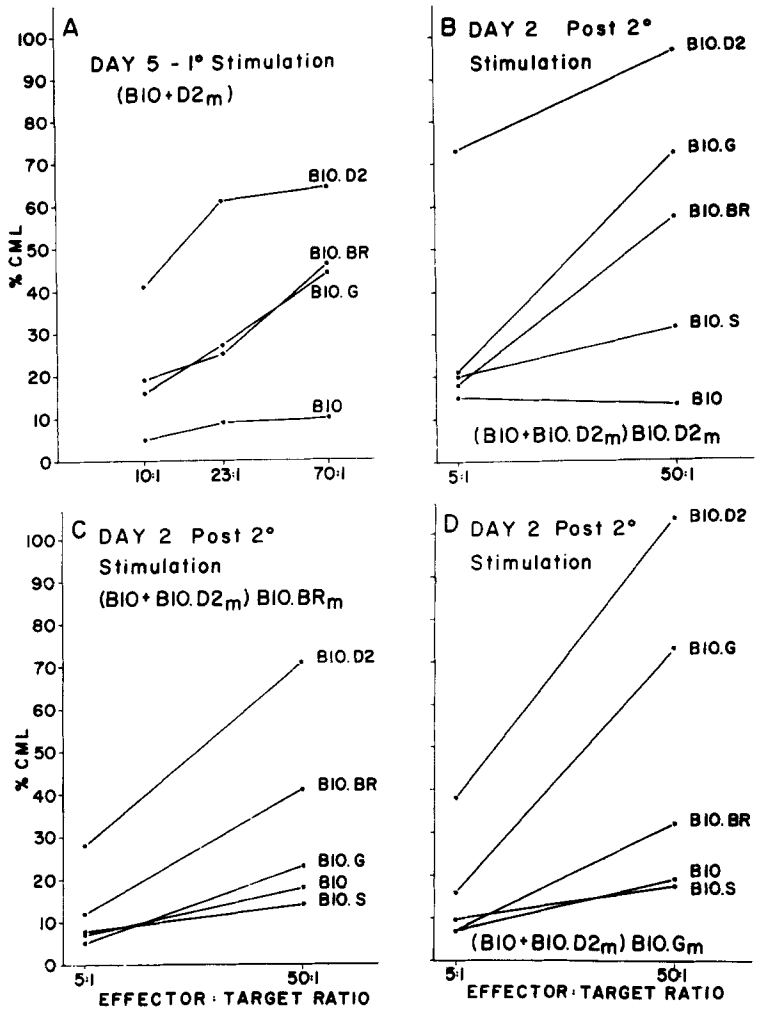

FIG. 3. Secondary stimulation of B10 spleen cells sensitized to mitomycin C-treated B10.D2 spleen cells by strains whose SD antigens cross-react with those of B10.D2 as determined by cross-killing in the day 5 primary CML response. Fig. 3 A shows the percent CML on the four given target cells (B10.D2, B10.BR, B10.G, and B10) on day 5 after primary sensitization. Fig. 3 B-D show the cytotoxicity on the same four target cells plus B10.S 2 days after restimulation on day 14 with B10.D2$_m$ spleen cells (B), B10.BR$_m$ spleen cells (C), and B10.G$_m$ spleen cells (D). CML results were obtained 2 days later against the same four target cells plus B10.S.

and cooperates (LD-reactive PHCs) in the generation of cytotoxic lymphocytes raises certain questions regarding possible roles of these two populations in the secondary response (16–19).

Highly specific and efficient CTLs are generated after secondary stimulation. The restricted specificity of this response is probably based in part on a suppressor mechanism generated in MLC (20). In terms of positive selection in the primary MLC, two possibilities for the cellular basis of the in vitro secondary

response can be considered. It may be that incubation of the cells beyond their initial proliferative and cytotoxic peak allows the development of independent "memory" cells which are qualitatively different from the cells responding to alloantigens in the primary MLC-CML culture. Alternatively, it may be that during the sensitization phase, the cells which have responded by differentiation and proliferation simply survive, preferentially leading to an increased percentage of specifically responding cells; the magnitude, kinetics, and specificity of the secondary response would reflect this form of in vitro selection. We shall refer to the lymphocytes responding rapidly upon restimulation as secondary lymphocytes without implying a prejudice as to the mechanism on which this response is based.

Extensive cytotoxicity is generated after secondary stimulation. One can only assume that these are secondary CTLs under one of the two mechanisms just discussed. The question whether there are also secondary PHCs that respond on restimulation is not clear although the data presented in this paper is consistent with the existence of such cells based on the extensive early proliferative response that may be analogous to the primary response. In fact, we would propose the following model to account for restimulation of the cytotoxic response.

After primary MLC sensitization both secondary PHCs and secondary CTLs are generated. In the primary sensitization phase only the CTL precursors reactive to those SD antigens present might be activated; all other CTL precursors reactive to SD antigens not present on the stimulating cells would be selected against, and therefore are not present at the time of restimulation.

For the primary CML response, the CTL may require two signals for activation. Signal one by the SD antigen leading to the specificity of the CML response; signal two possibly given by the product(s) of the LD-stimulated PHC. We would hypothesize that in the secondary response, the CTLs responsive to the SD antigen present on the primary stimulating cell are "poised" to differentiate rapidly and may require only the cooperative signal two to be activated. However, while this may even be the prime mechanism for activating the CTLs in the secondary, the administration of signal one to the CTLs in the secondary may either increase the probability that the particular CTL in question will be triggered by signal two or may trigger that CTL alone. Other models to explain the data we have presented are discussed below.

A role for the LD stimulus that fits our model is demonstrated in experiments where the primary stimulating population differed from the responder by LD and SD antigens. In the restimulation phase, LD differences alone were able to reactivate the specific anti-SD cytotoxic response (Fig. 2). Although we do not add the SD antigen upon restimulation in such experiments, it is possible that the SD antigens have, in some form, remained in culture since primary sensitization. The SD antigens may however not be needed in the secondary culture. Secondary CTL differentiation may, as discussed, require only the cooperative signal from LD-reactive PHCs or some substitute for this signal. Further, the relationship between the amount of PHC collaborative stimulus generated and the amount of CML may not be strictly linear but rather based on some needed threshold level of help. This would explain how LD-cross-reacting restimulating

cells, presumably bearing only some of the LD determinants to which cells were sensitized in the primary MLC, were able to induce the same level of CML activation to the original stimulating SD antigens as restimulating cells bearing the LD antigens present on the initial stimulator (Fig. 2). In man the magnitude of the proliferative response in secondary stimulation correlates with shared LD determinants; this has been used as a method for LD typing (4).

Our findings could also be explained by cytophilic transfer of the LD antigen receptors to the specific SD-reactive cytotoxic cells in the primary culture such that the LD difference alone will activate these cells in the secondary (5). Other mechanisms by which LD differences could activate CTLs directly are also possible.

Restimulating SD antigens do affect the specificity of cytotoxicity generated in secondary cultures (Fig. 3). This is observed best when third-party cells that bear cross-reacting SD antigens with the primary stimulating cells, restimulate cytotoxic activity that "deviates" toward targets from that third party. If within the poised population of CTLs, different subpopulations exist, each directed against specific SD determinants on the primary stimulator, but those subpopulations that also recognize the shared (or cross-reacting) SD antigens on the third-party restimulating cells are most strongly activated, this would account for the deviation of the response toward the SD antigens present on the cross-reacting restimulating cell. A suppression mechanism to account for deviation is also possible. The specificity of this deviation (Fig. 3 C and D) indicates that the shared antigens recognized by B10 plus $(B10.D2)_m$ CTLs on B10.G targets are distinct from the shared antigens recognized on B10.BR. This is directly testable by CML-blocking experiments, currently in progress, using unlabeled "cold" cells in the CML assay. Preliminary blocking results in man suggest that the killing of third-party targets is mediated by recognition of antigens shared by the third-party and primary stimulator, rather than antigens unique to the third party (21). The LD helper effect, presumably activated by cross-reacting LD antigens on the third-party cells would promote the development of all cytotoxic cells present in the culture after primary sensitization. Since the early secondary CML after restimulation would in all cases involve those CTLs which are active against the original sensitizing cell, this would explain the retention of maximal CML against that cell.

It is not yet clear whether the killing directed at the LD antigens (Table I) is mediated by CTLs belonging to the same functional subpopulation of T cells responsible for anti-SD killing in CML. (We recognize that the CML target in these cases might not be the LD antigens per se but rather other antigens determined by closely linked loci.) It is our supposition that a distinct subpopulation of LD-reactive CTLs should be separable from the majority of LD-reactive cells which function as PHCs. The arguments and model we have presented above can thus be extrapolated to these experimental results. The cellular basis of the secondary response may be best clarified by protocols using monolayer adsorption or anti-Ly antisera.

We have recently published results which we interpret to show that the primary response is probably initiated by the SD antigens and that the LD response allows the expansion or differentiation of the anti-SD cytotoxic re-

sponse (9), in analogy with the model proposed by Dutton and Hunter (22) and Schimpl and Wecker (23) for the development of the antibody-forming response and the role of T helper cells in that system. We extend this model to the secondary-type response based on the results presented in this paper and postulate that the LD-responsive PHC is present in the secondary cultures and responds, again primarily to LD. Further, similar to the primary, the LD helper effect permits reactivation of the SD-specific CTLs.

Summary

Lymphocytes stimulated in mixed leukocyte cultures and left for 13–17 days, i.e. beyond their peak proliferative and cytotoxic reactivities, can be restimulated to give a secondary-type rapid and strong proliferative and cytotoxic response when confronted with cells of the original sensitizing cell donor. We have concerned ourselves primarily with the requirements of restimulation for the presence of LD and/or SD stimuli on the restimulating cells. *(a)* The low level cell-mediated lympholysis (CML) associated with LD differences in a primary CML can be restimulated to give a secondary-type response by those same LD antigens. *(b)* If the original sensitizing cells differ from the responding cells by both LD and SD antigens, restimulation with only the LD antigens, or third-party cells presumably carrying cross-reactive LD antigens, can restimulate the secondary CML response directed against the SD antigens on the original sensitizing cells. *(c)* The presence of SD antigens on the restimulating cells that are cross-reactive with the primary sensitizing SD antigens (as determined in a primary CML) leads to the preferential activation of cytotoxic T lymphocytes reactive to those antigens although maximum cytotoxicity is still directed at cells carrying the original sensitizing SD antigens. A model to explain these results is presented.

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