ANTIGEN PRESENTATION TO HUMAN T LYMPHOCYTES

I. Different Requirements for Stimulation by Hapten-modified Cells vs. Cell Sonicates*

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It is generally thought that macrophages present antigen on their surfaces to elicit T cell-mediated functions such as proliferation to soluble antigens (1–3) and priming of helper cells for antibody responses (4, 5). Several reports have demonstrated that macrophages pulsed with soluble protein antigens and immune T cells interact to form clusters in vitro (6, 7). Data from mouse (8), guinea pig (9), and human (10, 11) studies suggest that specific physical interactions between antigen-presenting cells and primed responders permit antigen recognition in the context of Ia molecules. Recent studies in man have illustrated a requirement for HLA-D identity between priming and restimulating macrophages for optimal proliferative responses to soluble antigens (12, 13). Antigen presentation, however, may not be exclusively a function of macrophages. Other Ia^+ cells may effectively present antigen and thereby induce immune responses (14, 15). The chemical nature and form of the antigen, whether soluble or on a cell surface, could also influence cellular requirements. In this paper, we probe the requirements for induction of secondary T cell proliferation to intact hapten-modified cells as compared with cell sonicates. Our interest centers on defining the immunogenic unit recognized by primed T cells, as distinct from any accessory cell requirements for stimulation by these two forms of antigen. Using Ia^+ and Ia^- peripheral blood mononuclear cells (PBMC),[1] we have observed that for secondary responses, Ia molecules are required on the hapten-modified cells, but that macrophages are not needed for either hapten presentation or other accessory functions. In contrast, functions performed by macrophages may be essential for responses to hapten-modified autologous cell surface proteins presented as sonicates. Thus, Ia^+ macrophages, although they perform a role in processing and/or presenting bound membrane fragments, are unnecessary when intact hapten-modified Ia^+ lymphocytes are used as the antigen carriers. Our data further suggest that stimulation occurs only when hapten-modified proteins and Ia are presented on the same cell surface.

Materials and Methods

Cell Culture Techniques. Leukocytes were obtained from healthy donors by leukapheresis. PBMC were isolated as previously described (16) and frozen in RPMI 1640 (Grand Island

* Supported in part by grants AI15394 and RR00350 from the U. S. Public Health Service Research.

[1] *Abbreviations used in this paper:* AMLR, autologous mixed lymphocyte reaction; FL, fluorescein; IL-2, interleukin 2; MLR, mixed leukocyte reaction; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; TNP, trinitrophenyl.

Biological Co., Grand Island, N. Y.) with 1.5% bovine serum albumin (Cohn Fraction V; Sigma Chemical Co., St. Louis, Mo.), 25 mM Hepes buffer (Microbiological Associates, Bethesda, Md.), 25 U/ml heparin sodium (Fisher Scientific Co., Pittsburgh, Pa.), and 7.5% dimethyl sulfoxide, using a controlled-rate freezer. Such cells stored in a liquid nitrogen freezer for up to 1 yr are effective both as responders and stimulators for hapten-induced proliferative responses (16). Conjugation of cells with the haptens trinitrophenyl (TNP) and fluorescein (FL) and culture techniques have been previously described in detail (16). Cells were cultured in RPMI 1640 supplemented with 20% AB-positive, heat-inactivated, pooled human plasma. For primary stimulation, cultures were prepared in round-bottomed 17- × 100-mm tubes (2001; Falcon Labware, Oxnard, Calif.). Unfractionated responder and hapten-modified, 500-rad-inactivated stimulator cells were cultured at equal concentrations of 7.5×10^5 cells/ml in 2 ml cultures for 21–28 d. Primed cells were then harvested, washed, E-rosetted twice, and placed into 0.2 ml cultures at 1.5×10^5/well with an equal number of 1,500-rad-inactivated, fresh, hapten-conjugated, or unconjugated stimulators. Proliferative responses were assessed by addition of 1.0 μCi of [^{3}H]thymidine (2.0 Ci/mM sp act; New England Nuclear, Boston, Mass.) to cultures for the final 20 h of the incubation period.

Data from separate experiments are expressed as mean counts per minute of incorporated [^{3}H]thymidine from three or four replicate cultures with the standard error of the mean. Net counts per minute were calculated by subtracting the counts per minute of responses to unconjugated stimulators from the counts per minute of cultures with hapten-conjugated stimulator cells. E/C (experimental/control) is defined as counts per minute from cultures containing hapten-conjugated stimulator cells divided by counts per minute from those with unconjugated stimulators.

Cell Sonicates. Sonicates of hapten-modified and unmodified 1,500-rad-inactivated PBMC subpopulations were prepared with a probe sonicator (Biosonic II sonifier; Wills Scientific Co., Rochester, N. Y.), similarly to methods described by Heber-Katz and Shevach (17). After washing three times in phosphate-buffered saline (PBS), pH 7.4, hapten-modified cells were sonicated at 50% maximal output at a concentration of 5×10^7 cells/ml in PBS for 3 min at 4°C. The lysate was then promptly centrifuged at 1,000 *g* for 15 min to remove unbroken cells, the nuclear pellet, and other large membrane fragments. Supernates were centrifuged at 100,000 *g* for 75 min at 4°C, and the pellet was then resuspended at 1×10^7 cell equivalents/ml and used fresh or stored at −70°C until use. For stimulation of primed lymphocytes, 1×10^6 cell equivalents were added to each 0.2-ml microtiter well. Accessory cells, when added, were treated with 1,500 rad.

Cell Fractionation Procedures. PBMC were separated as previously described into macrophage, non-T lymphocyte, and T lymphocyte subpopulations (18). T cells were >92% E-rosette$^+$, <2% surface immunoglobulin$^+$, <0.3% esterase$^+$, and <5% Ia$^+$. Non-T lymphocytes, after double passage over Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, N. J.), were <0.3% esterase$^+$ and 65–70% Ia$^+$. Macrophages were >95% nonspecific esterase$^+$. Ia$^-$ (or perhaps more precisely Ia^{low}) macrophages were prepared by treatment with monoclonal anti-Ia (L243) plus complement (Low-Tox-H rabbit complement, lot 3331; Accurate Chemical and Scientific Corp., Hicksville, N. Y.), as previously described (16), and resulted in <5% Ia$^+$ cells. Ia$^+$ cells were determined by indirect immunofluorescence with monoclonal anti-Ia (L203). Cell suspensions were incubated for 1 h at 23°C with L203 antibody in PBS with 0.1% NaN_3 (to prevent cell surface antigenic modulation) and 1% bovine serum albumin. Cells were stained with FL-conjugated goat anti-mouse κ chain (Gateway Immunosera Co., St. Louis, Mo.), washed several times, and enumerated by fluorescence microscopy. L203 and L243 hybridoma antibodies were generous gifts of Dr. Ronald Levy, Howard Hughes Medical Institute Laboratory at Stanford University Medical Center, Stanford, Calif.

Results

Ia-bearing Lymphoid Subpopulations Can Stimulate Primed Cells in the Absence of Macrophages. Previously, we examined the secondary proliferative responses to PBMC subpopulations that were hapten modified. All subpopulations bearing Ia determinants could elicit secondary hapten-specific proliferative responses, whereas those that

did not bear Ia were ineffective in this regard (16). A question that remained, however, was whether unmodified macrophages were required for this response as accessory cells (e.g., for elaboration of interleukin 1) or as antigen presenters. The primed responders contained ~10% esterase$^+$ cells; these were enlarged cells not typical of resting monocytes, and the transfer of antigen from TNP-modified stimulators to such macrophages had not been ruled out. Therefore, primed cells were E-rosetted twice to remove these cells and restimulated with the subpopulations listed in Fig. 1. It is clear that non-T lymphocytes were highly effective stimulators, even in severely macrophage-depleted cultures (<0.3% esterase$^+$ cells). Thus, we were unable to demonstrate an intrinsic requirement for macrophages in secondary cultures, either for antigen presentation or other accessory cell functions. Moreover, adding back graded numbers of macrophages did not increase the response; in fact, macrophages were somewhat suppressive at higher doses. Dose-response data in our previous studies (16, 18) demonstrated that the hierarchy of proliferative responses induced by subpopulations was similar at several cell concentrations (from 1×10^4 to 2×10^5 per well). Non-T cells were always more effective stimulators than PBMC or monocytes.

Fig. 1 also demonstrates a second interesting observation. The proliferative response to unmodified non-T lymphocytes was consistently greater, often three- to fivefold, than that to macrophages or unfractionated cells, indicating that esterase$^-$ non-T lymphoid cells were potent stimulators of an autologous mixed leukocyte reaction (AMLR) after antigen priming.

Kinetics and Hapten Specificity for Responses to Sonicates. To compare antigen presentation requirements for hapten coupled to intact cell surfaces vs. hapten-modified membrane fragments, optimal response conditions were defined first. Initial experiments, as shown in Fig. 2, demonstrated that the kinetics of proliferation to cell

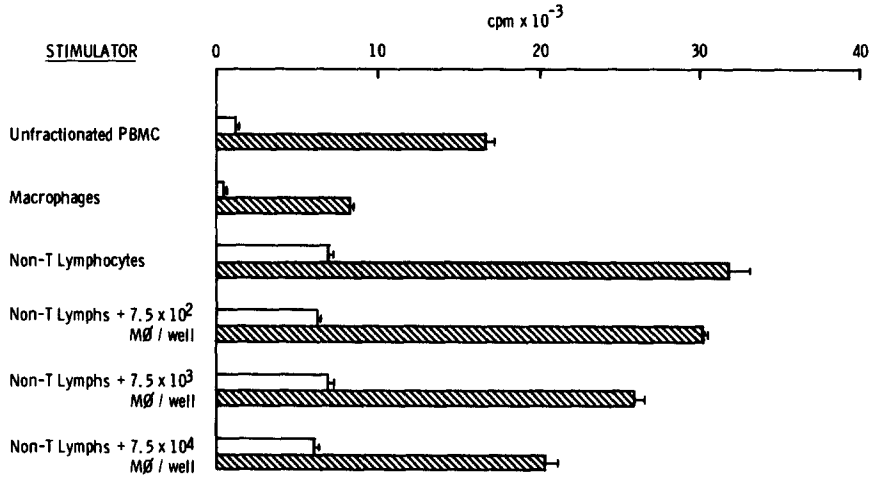

FIG. 1. Hapten-modified stimulator cells do not require added fresh macrophages. TNP-modified (▨) or unmodified (□) stimulators of each subpopulation noted were added to primed responder cells that had been E-rosetted twice immediately before restimulation. The same method was used to prepare responder cells in subsequent experiments. Nonspecific esterase staining values in this experiment were: primed responders, 0.27%; unfractionated PBMC, 18%; macrophages, 95%; and non-T lymphocytes, 0.31%. Stimulators were added to primed responder cells on day 21 and proliferation was assessed 3 d later.

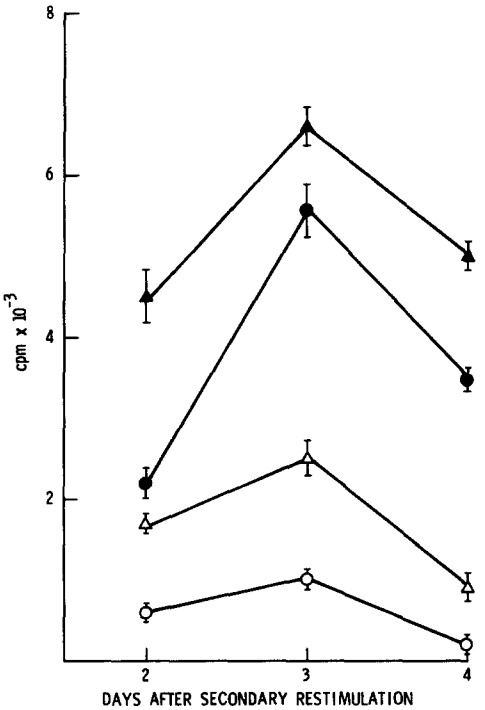

FIG. 2. Kinetics of secondary proliferative responses to sonicates. Stimulators were macrophage (Δ, ▲) and unfractionated PBMC sonicates (○, ●). The closed symbols represent TNP-modified sonicates and the open symbols represent unmodified sonicates. In all cases, 3×10^4 unmodified macrophages per well were added.

sonicates followed the same pattern as stimulation by intact hapten-modified cells, with peak responses 3 d after restimulation. Thus, in subsequent experiments, proliferation was assessed on day 3. In addition to those sonicates shown in the figure, sonicates of hapten-modified non-E-rosette and E-rosette cells followed similar kinetics. Hapten specificity of the response to sonicates was assessed by priming responder cells to TNP- or FL-modified cells (Table I). TNP-primed cells proliferated in a secondary response only to TNP cells or TNP sonicates, whereas FL-primed cells showed similar specificity for FL-derivatized cells and sonicates. In dose titration studies, a sonicate concentration of 1×10^6 cell equivalents per well appeared optimal (data not shown). Significant proliferation was induced by sonicate preparations (E/C ranging from 3 to 5) as compared with hapten-modified cells (E/C about 6). However, responses to sonicates by net counts per minute analysis did not exceed 30% of those observed with unfractionated hapten-modified cells.

Sonicated Membrane Preparations of Lymphoid Subpopulations Require the Addition of Fresh Macrophages to Stimulate Primed Cells. Fig. 3 illustrates data from experiments to determine whether proliferation to sonicates was dependent on macrophages, as has been described with soluble antigen systems (1–3). If so, this would be in contrast with the data in Fig. 1 for intact modified stimulators. When cells primed to unfractionated hapten-modified cells were stimulated by cell sonicate preparations, as opposed to intact cells, significant secondary responses were observed only when fresh unmodified macrophages were added. The addition of 3×10^4 macrophages to

TABLE I

*Hapten Specificity of Proliferative Response to Cell Sonicates**

Responder	Stimulator	cpm ± SEM	Δcpm
Primed to TNP-PBMC	PBMC	3,427 ± 203	0
	TNP-PBMC	19,524 ± 621	16,097
	FL-PBMC	2,375 ± 107	<0
	PBMC sonicate	1,306 ± 101	0
	TNP-PBMC sonicate	5,092 ± 340	3,786
	FL-PBMC sonicate	1,263 ± 88	<0
Primed to FL-PBMC	PBMC	2,725 ± 215	0
	TNP-PBMC	3,376 ± 137	651
	FL-PBMC	13,717 ± 493	10,992
	PBMC sonicate	1,208 ± 72	0
	TNP-PBMC sonicate	1,114 ± 95	<0
	FL-PBMC sonicate	4,632 ± 310	3,424

* Primed responders and stimulators were co-cultured as previously described. 3×10^4 unmodified macrophages were added per well for sonicate responses. Proliferation was assessed 3 d later.

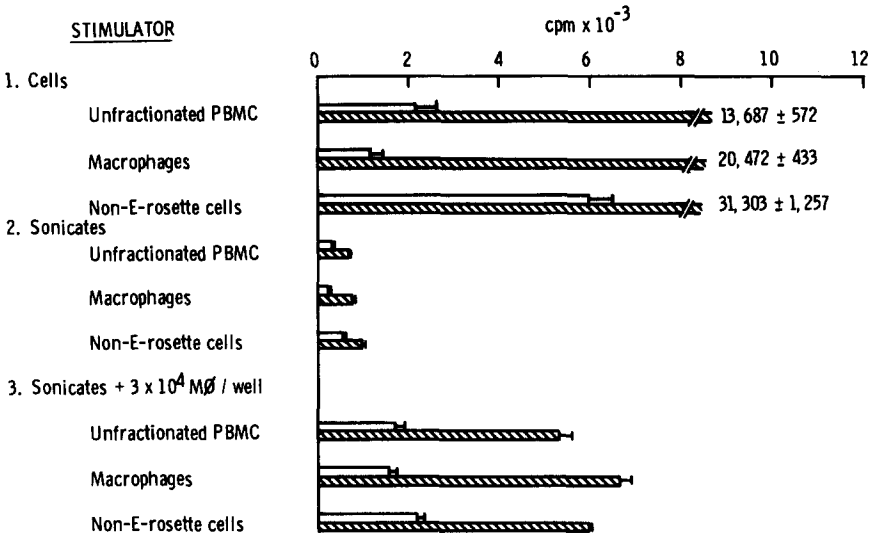

FIG. 3. Efficacy of hapten-modified cell sonicates compared with intact cells as stimulators of secondary proliferative responses. TNP-modified (▨) or unmodified (□) stimulator cells or sonicate preparations from each subpopulation were added to primed responder cells E-rosetted twice on day 21; proliferation was assessed 3 d later.

each well containing primed E-rosetted responders plus sonicates resulted in ~20% esterase$^+$ cells per well. This resembled the esterase$^+$ cell count in fresh unfractionated PBMC. Interestingly, despite the clearly significant hapten-specific response to sonicate antigens only after macrophage reconstitution, the magnitude of the response to intact unmodified non-E-rosette cells equalled or exceeded the responses to hapten-modified sonicates. In contrast, unmodified macrophages and unfractionated cells induced minimal proliferation, similar to unmodified cell sonicates plus 3×10^4 macrophages/well, providing additional evidence for a marked AMLR to non-T cells after priming to haptenated self cells.

Ia$^+$ and Ia$^-$ Sonicated Membranes Can Stimulate Primed Cells. The question of the

stimulatory moiety in the cell sonicate preparation was examined through subpopulation analysis. The possibility that hapten-modified Ia molecules were directly responsible for stimulation was tested by using sonicates of Ia^+ vs. Ia^- cells (Fig. 4). Again, added macrophages were required for secondary responses, but it is clear that sonicates of Ia^- cells (T cells and Ia^- macrophages) stimulated primed cells nearly as well as sonicates from Ia^+ cells. Thus, hapten-modified self proteins presented as membrane fragments, regardless of the presence of hapten-modified Ia, were able to stimulate secondary responses. More importantly, although both Ia^+ and Ia^- cells were efficacious as sources of hapten-modified cell sonicates, the requirement for macrophages in sonicate induction of such responses was subserved best by the Ia^+ macrophage subpopulation. Ia^- macrophages were clearly and consistently less effective in this role. Minimal proliferation could be attributed to low levels of Ia, not visualized by immunofluorescence, still remaining on these cells.

An Accessory Cell Required for Secondary Responses to Sonicates Is Present in the Ia^+ Macrophage Preparation but Not in Ia^+ Non-T Lymphocytes. Ia molecules clearly play an important role in secondary proliferative responses to hapten-modified cells. When hapten was directly conjugated to a cell surface, only those cells that expressed Ia molecules stimulated primed cells to proliferate. We further demonstrated with sonicates that hapten-modified Ia was not required. We concluded, therefore, that Ia molecules were somehow involved in either antigen recognition or in cell interaction inducing activation. Whether Ia alone was sufficient or other macrophage properties were also required remained to be determined. Fig. 5 demonstrates again that macrophages could serve as accessory cells in allowing sonicates to stimulate proliferation of primed cells. In contrast, non-T lymphocytes, which, when used as hapten-modified intact cells were the best cell stimulators, and when unmodified were the most potent stimulators of AMLR, could not function as accessory cells for sonicate responses. Thus, sonicate binding and possibly processing for presentation to primed cells may be functions only carried out by macrophages. Furthermore, as previously demonstrated (Fig. 4), only Ia^+ macrophages were effective in this regard.

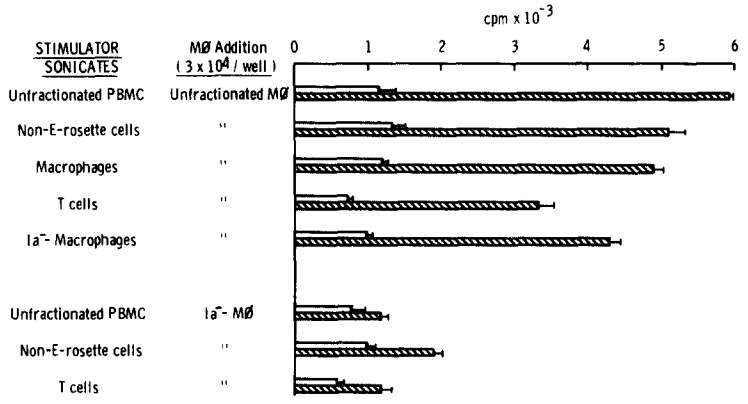

FIG. 4. Efficacy of Ia^- cell sonicates in stimulating secondary proliferative responses in the presence of Ia^+ macrophages. TNP-modified (▨) or unmodified (□) stimulators were added to primed responders and proliferation was assessed 3 d later.

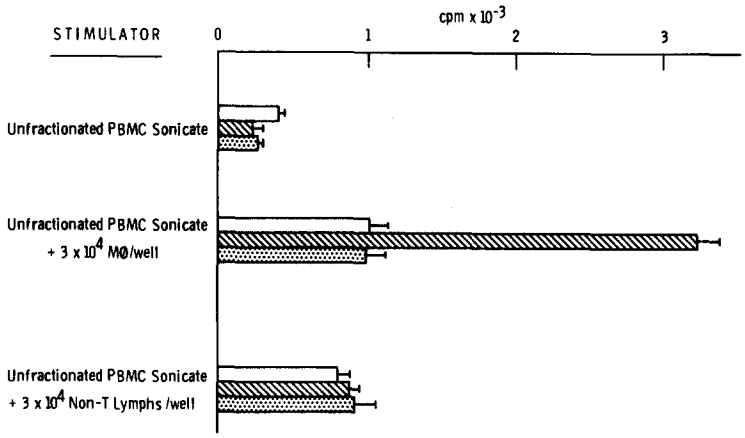

FIG. 5. Ia^+ non-T lymphocytes cannot stimulate proliferation of primed cells to sonicates. TNP-modified (▨), FL-modified (▩), or unmodified (□) stimulators were added to twice E-rosetted primed responders and proliferation was assessed 3 d later.

Discussion

The functions of macrophages in cell-mediated and humoral immunity include antigen presentation and production of factors that stimulate or regulate other cells. The present experiments directly focus attention to two separate issues in antigen presentation: first, the role of hapten-modified antigen-presenting cells in definition of the immunogenic moiety recognized by primed T cells; second, the apparent differences between certain subpopulations of potentially effective antigen presenters in displaying hapten-modified antigens not intrinsically attached on their surface. We have demonstrated distinct differences between macrophage requirements for secondary proliferative responses to intact hapten-modified cells and to hapten-modified autologous cell fragments. It appears that although hapten-modified cells bearing Ia antigens are essential for recognition by primed T cells, macrophages are not intrinsically required for a proliferative response. Antigen presentation by macrophages is not needed for hapten-modified cell surface moieties, perhaps because these are bound already and do not require attachment to such antigen presenters. In contrast, in order for sonicates to be antigenic, binding and possibly processing may be required. Our data demonstrate that Ia^+ macrophages, unlike Ia^+ non-T lymphocytes, can fulfill this function. In fact, a subpopulation of macrophages bearing Mac-120, a determinant recently described by Raff et al. (19), may be essential. Mac-120^+ cells have been shown to be necessary for proliferative responses to soluble antigens and mitogens (19, 20). It is also important to note that Steinman et al. (21) have defined another accessory cell isolated from murine adherent cells, the dendritic cell, for which a human analogue has not yet been identified. These strongly Ia^+ cells are, by themselves, the most effective stimulators of the MLR (22) and syngeneic MLR (23), and could be a critical component of the stimulatory ability of macrophage or non-T lymphocyte preparations when hapten modified, although not when presented with sonicates. The latter is consistent with the observations of Steinman and Cohn (24) that dendritic cells, unlike macrophages, exhibit little or no endocytic activity toward antigens such as sheep erythrocytes and immune complex aggregates. Interestingly,

however, Sunshine et al. (25) reported that dendritic cells were highly efficient in presenting soluble antigens to syngeneic T cells.

Through the use of TNP-modified soluble antigens in mice, Ballas and Henney (26) demonstrated that both major histocompatibility products and hapten needed to be expressed on the cell surface, but not necessarily covalently linked, in order to elicit secondary cytotoxic responses. Our data and another report on the murine system (17) support this concept for proliferative responses. Whether TNP sonicates are inserted or merely adsorbed to the cell surface of stimulator cells is unclear. Nevertheless, the presentation to primed cells required Ia-bearing macrophages. In eliciting secondary responses, this is a requirement that sonicates and soluble antigens share. On the other hand, macrophages are not essential for stimulation by hapten-modified cells.

During the priming phase of both proliferative and cytotoxic responses, however, macrophages may be necessary. In primary responses, macrophage interleukin 1, as well as other lymphokines, may be required for recruitment of proliferating cells and stimulation of interleukin 2 (IL-2) production. Once expansion of antigen-reactive clones has taken place, restimulation of such clones may take place by presentation of antigen on Ia-bearing cells via the appropriate recognition unit. Primed T cells may then secrete IL-2 to facilitate enhanced T cell proliferation. Although our data do not directly address this issue, macrophages or macrophage products were not needed in the secondary response. Recently, IL-2 has been shown to be a product of the AMLR in mice (27). Consequently, it is important to note that simply the provision of hapten-modified cell sonicates plus a putative IL-2 signal (Ia^+ non-T lymphocytes) was not sufficient for induction of secondary hapten-specific proliferative responses to sonicates. The cellular requirements for stimulation of IL-2 from primed vs. unprimed cells have not been defined, however, and all Ia^+ cells may not suffice. In addition, the ability of certain sets of Ia^+ cells to bind or present antigen may vary considerably. Both immunogenic presentation and IL-2 production may, therefore, be essential for proliferation.

The apparent strict requirement for macrophages in some systems may reflect several tissue culture variables. Recently, Geier and Cresswell (28) reported that human primary MLR responses were macrophage independent at higher cell densities (a total of 2×10^5 cells/well, $<0.05\%$ $esterase^+$) and that this requirement was also influenced by the shape of the culture vessel; i.e., round- or flat-bottomed. These data support our observation that macrophages are not intrinsically necessary for a proliferative response to cell surface antigens. In the case of higher density cultures, antigen presentation via cell-cell contact would be facilitated and proliferation would be enhanced, assuming that the appropriate recognition units were displayed.

Although we tried using Ia^- macrophages as accessory cells in sonicate responses, and previously as hapten-modified cells (16), in neither case were they able to support hapten-specific proliferation. Although, as previously noted, it seems likely that Ia^- macrophage populations identified by fluorescent microscopy and cytotoxic antibodies may express a low level of Ia when examined by more sensitive techniques such as flow microfluorometry, the essential observation is that the procedures used in these studies clearly separated macrophages into two functionally distinct subpopulations. Such subpopulations may represent distinct lineages or different stages in monocyte/macrophage development. It is also possible that differences in production of soluble

factors as well as surface phenotype could account for the stimulatory ability of Ia^+ macrophages vs. the nonstimulatory character of Ia^- macrophages. This is an important issue that will demand future investigation. In addition, we have not yet studied the kinetics of possible reexpression of Ia after L243 plus complement treatment; nevertheless, our data clearly suggest that further expression of Ia is not taking place on these cells in the critical period necessary for development of secondary proliferative responses.

Although macrophages in culture have previously been considered necessary for responses to soluble antigens, the relative efficacy of other Ia^+ cells as accessory cells has not been clearly established. This has not been defined completely with regard to cell sonicates. Thus far, we have only examined non-T lymphocytes (65–70% Ia^+) and they are ineffective in stimulating proliferative responses with sonicates. Several investigators have reported that Ia^+ neutrophils (29, 30) and activated T cells (31) could at least partially restore the proliferative responses of purified T cells to selected soluble antigens. The ability of Ia^+ cells to bind or process particular antigens appropriately may be the critical factor.

It is important to note that Ia, if absorbed from sonicates, was not stimulatory on Ia^- macrophages, demonstrating that Ia molecules were either physically altered or not absorbed in sufficient quantity to reconstitute responses, or that intrinsically bound Ia molecules in an intact, normal membrane are required. The latter is suggested by Halper et al. (32), who demonstrated that leukemic B cells, although strongly Ia^+, could not stimulate an AMLR. Their data suggested a defect in membrane integrity of such cells, which they postulated interfered with appropriate antigen presentation. Furthermore, Thomas et al. (33) have shown that Ia^+ TNP-L_2C guinea pig leukemia cells only stimulate in the presence of Ia^+ macrophages. This was interpreted as a necessity for shedding and representation of TNP-modified L_2C antigens onto Ia^+ macrophages. Interestingly, concordant with our sonicate data, Ia^+ and Ia^- variants of TNP-L_2C cells were equally effective as stimulators, only in the presence of Ia^+ but not Ia^- macrophages.

In conclusion, the experiments illustrate a fundamental difference in the requirements for T cell recognition of cell sonicates vs. intact hapten-modified cells. Ia^+ and Ia^- sonicates were both stimulatory but only when presented on an Ia^+ macrophage. In contrast, hapten-modified Ia^+ cells may bypass a macrophage requirement and stimulate primed responders, whereas Ia^- cells will not. Studies of the priming process using hapten-modified soluble proteins of a homogeneous nature and hapten-modified cells will further clarify the antigen-presenting and/or accessory functions of human lymphoid cells.

Summary

We have investigated the cellular and antigenic requirements for incubation of secondary proliferative responses by human T lymphocytes. Two distinct properties of antigen-presenting peripheral blood mononuclear cells were studied: (*a*) the ability for appropriate cell surface constituents to construct an immunogenic moiety, and (*b*) the ability to present similar antigenic determinants when they are not covalently bound. Only Ia^+ hapten-modified cells were effective stimulators. In contrast, both Ia^+ and Ia^- cell sonicates could stimulate secondary proliferative responses, but only in the presence of an accessory cell. This accessory cell was present in Ia^+ macrophage,

but not in Ia^+ non-T lympocyte, preparations. In contrast, macrophages or soluble factors produced by macrophages were not required for primed T cells to undergo hapten-specific proliferation in response to hapten-modified Ia^+ stimulator cells. Thus, although all Ia^+ cells tested can stimulate primed cells to proliferate, not all Ia^+ cells can function as accessory cells for responses to sonicates. This may reflect the unique ability of a subpopulation(s) of Ia^+ cells to bind or process sonicates or soluble antigens for appropriate recognition by primed T cells.

We thank Ms. Mai Van for expert technical assistance, and Ms. Norma Olivarez and Ms. Patricia Mullally for excellent secretarial assistance.

Received for publication 4 May 1981 and in revised form 22 June 1981.

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