DIFFERENTIAL MACROPHAGE REQUIREMENTS FOR T HELPER CELL AND T HELPER CELL-INDUCED B LYMPHOCYTE PROLIFERATION*

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The immune response to most antigens involves three distinct cell populations: T and B lymphocytes and Thy-1$^-$, Ig$^-$, Ia$^+$ accessory cells. The analysis of the different phases in this process has been hampered by the very low frequency of specific cellular interactions occurring in cooperative responses, determined by the clonal frequencies of antigen-specific T and B lymphocytes. We have recently developed (1) in vitro systems allowing the simultaneous use of enriched populations from each of these three cellular sets with specificities such that specific cooperative interactions occur very frequently and encompass the majority of all cultured cells.

This experimental system of cell interactions was further developed to offer three remarkable advantages in the study of B-T-macrophage (Mϕ)[1] cooperation: (*a*) The raising of T helper cells against minor histocompatibility antigens polyclonally distributed on B cell surfaces allows for specific cooperative interactions with a large numbers (if not all) B cells, independent of their clonal specificity. Antibody responses, on the other hand, can be assayed polyclonally at the single-cell level. (*b*) The enrichment in long-term selective cultures and consequent cloning of helper T cells with such specificity provides homogeneous T cell populations, so that practically all lymphocytes seeded in culture interact specifically. (*c*) As the minor antigens specifically recognized by helper cells are also naturally expressed on Mϕ surfaces, this system bypasses the problems of antigen concentration and the steps of antigen uptake and processing, reducing the scope of the questions to the "stimulatory" properties of accessory cells.

We have now used this system to analyze the precise role of macrophages in T-B cell collaboration. The previous work in this area has centered on antigen processing and presentation by Mϕ to T cells, their participation in T cell activation, and the role of major histocompatibility complex (MHC) products in these phenomena. As a consequence, little evidence is available concerning B cell-Mϕ interactions, and the role of accessory cells in B cell triggering growth and maturation (for review, see 2 and 3). On the other hand, available experimental methods for Mϕ depletion, in

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[1] *Abbreviations used in this paper*: LPS, lipopolysaccharide; MHC, major histocompatibility complex; Mϕ, macrophage; PFC, plaque-forming cell; T_H, T helper.

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particular their selective removal from B cell populations, are quite unsatisfactory from a qualitative point of view. Furthermore, results of macrophage depletion have been difficult to interpret, as it has been impossible to distinguish between trivial "filler" effects and "specific" functions in cell interactions exerted by a very restricted number of macrophage cells.

In the present experiments, we compared the Mϕ requirements in the proliferative responses of helper T cells and B lymphocytes in the same cultures under conditions that confront roughly equal numbers of the two cell populations, homogeneous as to their interacting specificity, which are simultaneously responder and stimulator in functional terms. Although we are aware of the experimental limitations imposed by the methods for Mϕ depletion, the results obtained lead us to conclude that B cells, although naturally expressing antigen, cannot stimulate MHC-restricted helper T cell proliferation. In contrast, activated helper cells are shown to induce growth and maturation of resting B lymphocytes under the same conditions of Mϕ depletion.

Materials and Methods

Mice. C3H/HeJ (H-2^k), C3H/Tif(H-2^k), BALB/c(H-2^d), C57Bl/6 (H-2^b), B10.Br(H-2^k), and B10.D2(H-2^d) mice were from our colony, having been maintained on standard conditions. BALB.K(H-2^k) and BALB.B(H-2^b) mice were purchased from Olac, Bicester, England.

Reagents. Anti-Thy-1 monoclonal antibody: normal spleen cells were depleted of theta-positive cells by treatment with the rat IgM anti-Thy-1 monoclonal antibody J.I.J. (a kind gift of Dr. J. Sprent, University of Pennsylvania, Philadelphia, PA) at appropriate dilution, in the presence of rabbit complement. Lipopolysaccharide (LPS) from *Brucella abortus equi* (Difco Laboratories, Detroit, MI) was used in culture at a final concentration of 50 μg/ml.

G10 columns. Adherent cells were removed by two consecutive passages of anti-Thy-1 and complement-treated normal spleen cells over G10 columns (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the method of Ly and Mishell (4). Aliquots of 10^8 spleen cells were applied to a 5-ml column, and nonadherent cells were recovered after 30 min incubation at 37°C. The recovery from this treatment ranged from 20 to 30% of the cell input.

Cloned Helper T Cells. As described elsewhere (1), anti-minor T helper (T_H) cells were raised in C3H/HeJ mice by immunization with C3H/Tif spleen cells and enrichment in selective cultures for 6 wk before cloning in suspension cultures by limiting dilution on irradiated (2,000 rad) C3H/Tif spleen cells. The helper cell populations used here have been maintained over the last 16 mo by weekly subculture on irradiated C3H/Tif spleen cells.

Test Cultures. Microcultures were set up in triplicate on flat-bottomed microtiter plates in RPMI 1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, penicillin and streptomycin, glutamine, and 10% fetal calf serum. Each 0.2-ml culture contained 4×10^4 anti-Thy-1 and complement-treated C3H/Tif spleen cells and cloned helper T cells titrated in 1:3 step dilutions to a maximum of 2×10^4 per culture. When indicated, one or the other cell population was irradiated with 2,000 rad. Mϕ-rich populations were obtained by rinsing the peritoneal cavity with balanced salt solution supplemented with 5% fetal calf serum. These were anti-Thy-1 and complement treated, irradiated with 2,000 rad, and titrated in culture to a maximal concentration of 10^4 per well.

Assays. The cultures were assayed for proliferation (on day 3, if not indicated otherwise) by incubation with 1 μCi/well of [^{3}H]thymidine (TRA 310; The Radiochemical Centre, Amersham, England; sp act 2 Ci/mmol) for 4 h. B lymphocyte responses were also assayed by measuring antibody production, namely numbers of plaque-forming cells (PFC) in the protein A assay (5).

Results

MHC-restricted, Anti-Minor Helper Cells Induce Antigenic B Lymphocytes to Extensive Proliferation. In the present experiments, we have used clones of C3H/HeJ T cells

selected on the basis of their ability to activate normal C3H/Tif B cell populations, as measured by the appearance of polyclonal PFC in cooperative cultures (1). As expected from the polyclonal expression of antigen on C3H/Tif B cell surfaces, it was previously shown that these helper T cells induce a large fraction of B cells to proliferation and maturation. From the quantitative level of PFC response obtained, equal or superior to those induced by LPS, and the analysis of antibody specificities among IgM PFC, the polyclonality of the response was concluded (1). This has recently been confirmed in experiments determining the frequency of B cell clonal precursors that can be activated in limiting dilution conditions (unpublished results). Of particular importance in the definition of this system is the demonstration that B cell activation requires direct and specific recognition of antigen on B cell surfaces by helper cells, and that no soluble factors initiate the process of B cell activation (1, 6). Furthermore, the clones we have used in the present experiments are MHC restricted, as shown in Table I and Fig. 1. Thus, helper cell proliferative responses to irradiated stimulator cells, as well as cooperative induction of B lymphocytes, require that the "target" cells express H-2^k antigens. Other clones raised in the same conditions, as well as uncloned, enriched helper cell populations, however, can operate in a nonrestricted fashion (7).

Co-culture of helper cells with appropriate T cell-depleted normal spleen cells results in cooperative interactions and stimulation of both types of cells. As shown in Fig. 2a, the limiting factor in the proliferative responses of a fixed number of T cell-depleted target spleen cells is the number of specific helper T cells added to the cooperative cultures, i.e., the effector helper to target spleen cell ratios. Most interestingly, the magnitude of cell proliferation in these cultures is little affected by the irradiation of the inducer T cells, indicating that the bulk of the mitotic activity is due to target B cell proliferation, and that the effector functions of the helper cells used here is radiation resistant. In contrast, and as shown before (1), irradiation of T helper cells results in less than the optimal PFC development in the proliferating B cell populations (Fig. 2b). We conclude, therefore, that the number of helper T cells

TABLE I

*MHC Restriction of the Proliferative Responses of Anti-Minor Helper Cells to Antigenic Macrophages**

	Irradiated Mϕ‡	cpm/culture
Experiment 1	No	175
	C3H/Tif (H-2^k)	5,090
	C3H.SW (H-2^b)	696
Experiment 2	No	295
	B10.Br (H-2^k)	9,315
	B10.D2 (H-2^d)	1,581
	C57Bl/6 (H-2^b)	2,309
	B10.Br‡	156
	B10.D2‡	126
	C57Bl/6‡	75

* Proliferation was measured by a 4-h pulse with 1 μCi of [^{3}H]thymidine on day 3 of culture. All cultures contained 10^4 T_H cells.

‡ 10^4 anti-Thy-1 and complement-treated normal peritoneal cells irradiated with 2,000 rad.

§ No T_H responder cells.

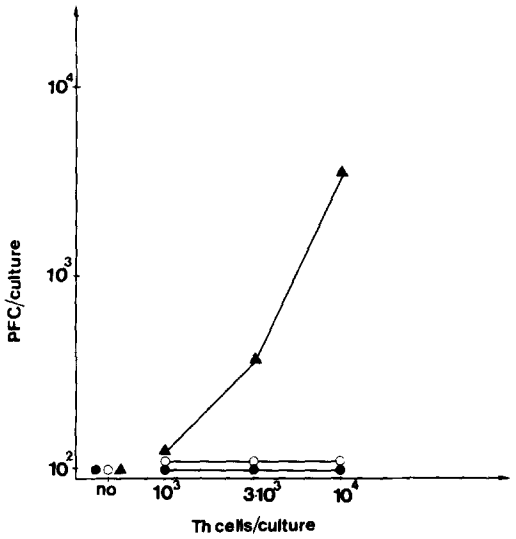

FIG. 1. Cooperative induction of B lymphocytes is MHC restricted. Cloned C3H/HeJ anti-C3H/Tif helper T cells were titrated in cultures of anti-Thy-1 and complement-treated spleen cells (4×10^4/culture) from B10.BR (▲), B10.D2 (●), and C57Bl/6 (○). Total Ig-secreting PFC were assayed on day 4.

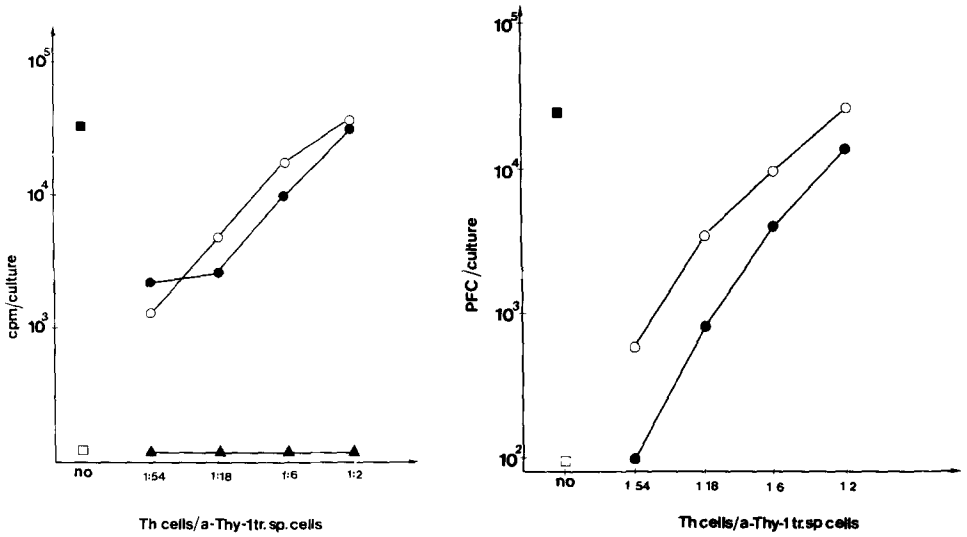

FIG. 2. (a) T cell-depleted C3H/Tif spleen cells (4×10^4/culture) were co-cultured with a titration of nonirradiated (○) or 2,000-rads-irradiated (●) helper T cells, at the indicated effector/target ratios, and the proliferative responses were measured on day 3. The proliferation of T cells (▲) and spleen cells (□) cultured alone as well as 50 μg/ml LPS (■) are indicated. (b) The same as in (a), but total PFC responses were measured on day 4 of culture.

initially added to cultures is sufficient to activate extensive B cell proliferation. These conditions allow us to study the requirements for proliferative responses of B lymphocytes independently of the mitotic activity of helper T cells. On the other hand, it

appears that maximal PFC responses, as compared with proliferative responses, require higher numbers of T helper cells.

Macrophage Depletion Abrogates Helper T Cell Proliferation. It has been extensively shown that macrophages play a crucial role in helper T cell function (2, 3). Macrophage T cell cooperation is MHC restricted (8), and it appears that Ia-positive macrophages are fundamental in stimulating T lymphocyte growth (9–11), confirming the results shown in Fig. 2a, indicating that the B cell population in our assay is responsible for the bulk of the mitotic activity in culture. Fig. 3 shows that helper T cells proliferate very little when stimulated with the numbers of spleen cells used in these experiments (4×10^4). Responses appear to be limited by the relatively low macrophage content of spleen. Thus, adherent cell depletion on Sephadex G10 columns completely abolished helper T cell proliferation, which is reconstituted by irradiated, anti-Thy-1 and complement-treated peritoneal cells. Reconstitution with 10^4 peritoneal cells results in helper cell responses markedly better than those obtained with 4×10^4 stimulator cells. A titration of macrophages into G10-passed spleen cell populations shown in Fig. 4 demonstrates that macrophages are in fact the limiting factor controlling helper T cell growth, and the very limited number of competent stimulator cells present in normal spleen. These results demonstrate that splenic B cells, in spite of expressing on their surface both antigen and restricting elements, are not competent in stimulating T cell growth, indicating that recognition of antigen in the correct Ia context does not constitute a growth signal to helper T cells. On a quantitative basis, the numbers of competent stimulator cells present in 4×10^4 anti-Thy-1-treated spleen cells correspond to those present in $\sim 10^3$ peritoneal cells. Furthermore, G10 column passage of the spleen cell populations leaves behind, if any

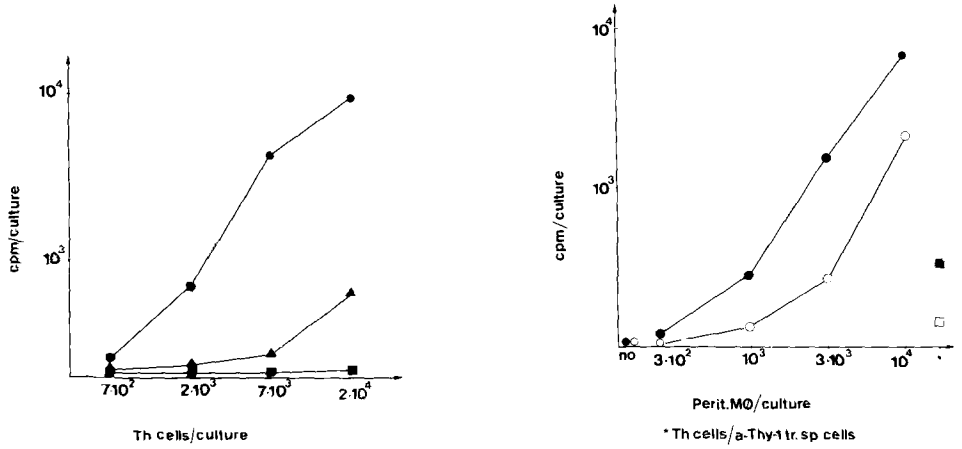

FIG. 3. The indicated numbers of helper T cells were cultured with 2,000-rad-irradiated C3H/Tif spleen cells (4×10^4), which were either simply anti-Thy-1 and complement treated (▲) or further depleted of adherent cells by passing them twice over G10 columns (■). Another set of cultures received, in addition to T cell-depleted, G10 passed, and irradiated spleen cells, 10^4 irradiated, anti-Thy-1 and complement-treated C3H/Tif peritoneal cells (●). All cultures were assayed for proliferation on day 3.

FIG. 4. Cloned helper T cells (2×10^4/culture [●, ■] or 7×10^3 cells/culture [○, □]) were cultured with the indicated numbers of 2,000-rad-irradiated, T cell-depleted peritoneal cells (●, ○), or with 4×10^4 irradiated, T cell-depleted spleen cells (■, □), and the proliferative responses measured on day 3 of culture.

stimulator cells at all, fewer than those present in 300 peritoneal cells, in numbers that are insufficient to stimulate any significant helper cell proliferation.

Helper Cell-induced B Cell Proliferation Is Not Abolished by Macrophage Depletion. Because, as shown above, B cell proliferation can be induced by nonproliferating helper cells, we could assess the requirements for macrophages in those responses. Fig. 5 presents one such experiment on the influence of G10-adherent cells on B cell growth. Normal spleen cells were first treated with anti-Thy-1 and complement and subsequently passed twice over Sephadex G10 columns. Such enriched B lymphocyte populations were either irradiated and assessed as stimulators of helper cell proliferation or not irradiated and used as responders to the mitogenic activity of helper cells. T cell growth was completely abrogated by G10 passage, confirming the results above and providing an internal, quantitative control for accessory stimulator cell depletion. It follows that all proliferation observed in cultures of helper cells and G10-passed spleen cells must be due to B lymphocyte proliferation in the target cell population. In contrast to the abrogation of helper cell responses, T_H cell-induced B cell growth, although somewhat reduced, is still very significant after G10 passage of spleen cells. Although these experiments cannot provide qualitative conclusions, as we cannot be sure of complete macrophage removal from the B cell-enriched populations, they demonstrate a remarkable quantitative difference in macrophage requirements in T_H cells and T-induced B lymphocyte growth, respectively. To definitely ascertain that all proliferation detected in cooperative cultures of G10-passed spleen cells was due to growth of the target B lymphocytes, we have repeated these experiments using helper cell populations that were irradiated before culture. This protocol also included the reconstitution of the depleted spleen cell populations with T cell-depleted, irradiated

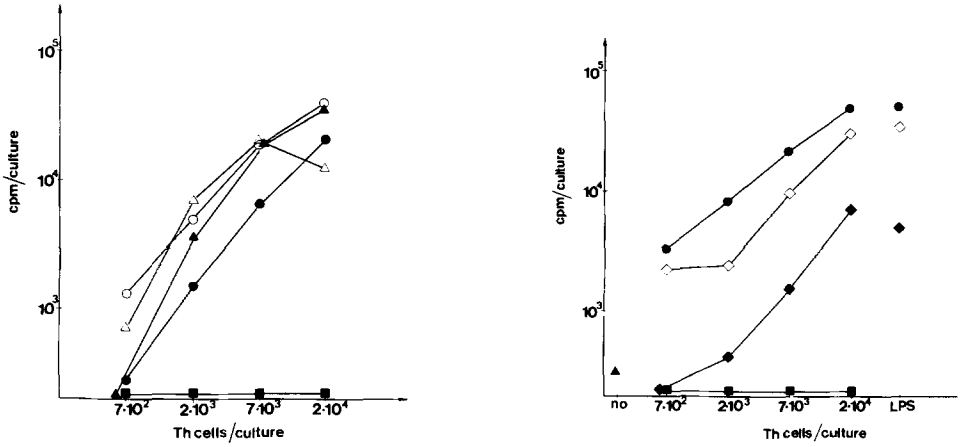

FIG. 5. T cell-depleted C3H/Tif spleen cells (4×10^4) before (△, ○) or after (▲, ●) two passages over G10 columns, were co-cultured with the indicated numbers of cloned helper T cells. Proliferative responses were measured day 3 for this as well as for cultures of the same numbers of T cells with 2,000-rad-irradiated T cell-depleted and G10-passed spleen cells (■). Two independent experiments (△, ▲; ○, ●) are shown.

FIG. 6. Day 3 proliferative responses induced by the indicated numbers of 2,000-rad-irradiated T helper cells or LPS in T cell-depleted C3H/Tif spleen cells (4×10^4/culture) before (◇) and after (◆) two passages over G10 columns, and after reconstitution of G10-passed cells with 10^4 irradiated, T cell-depleted peritoneal cells (●). Thymidine uptake in cultures of nonirradiated T cells with irradiated T cell-depleted and G10-passed spleen cells is also indicated (■).

peritoneal cells. As shown in Fig. 6, although somewhat more reduced than upon stimulation by nonirradiated helper cells, a significant proliferation of G10-passed spleen cells induced by irradiated helpers is still observed, under conditions of accessory cell depletion that result in no helper cell proliferation at all. Reconstitution of the cooperative cultures with large numbers of peritoneal cells results in enhanced proliferation at levels higher than before G10 column treatment, indicating an important, although not essential role of macrophages in this process. This enhancement could be due to three distinct reasons: (*a*) nonspecific improvement in culture conditions; (*b*) activation of higher levels of effector helper activity in the T cell population; (*c*) direct and specific macrophage participation in B cell proliferation. These will be considered below in some detail. Interestingly, the LPS-induced proliferative response was drastically reduced after G10 passage, and it could be fully reconstituted by addition of irradiated T cell-depleted peritoneal cells. The reduction in LPS responses, therefore, cannot be solely ascribed to loss of LPS-reactive B cells in the G10 columns, and it indicates a clear macrophage dependence of these responses. It remains to be shown whether the same B cell population responds to LPS in both sets of conditions and whether macrophage effects are specific (e.g., production of necessary growth factors) or pleotrofic (e.g., filler effects). In any case, these results confirm a very extensive macrophage depletion and point out that helper cell-induced B cell proliferation, if not completely independent of accessory cells, is certainly less dependent than LPS-induced responses. We conclude that helper cell-dependent B cell proliferative responses are quite independent of Mϕ, in particular when the helper cell population had not been irradiated before culture. On quantitative levels, these responses are clearly less dependent on adherent accessory cells than LPS-induced proliferative responses.

Helper Cell-induced B Cell Maturation to PFC is Relatively Independent of the Presence of Macrophages. Similar protocols using nonirradiated helper cells were applied to study

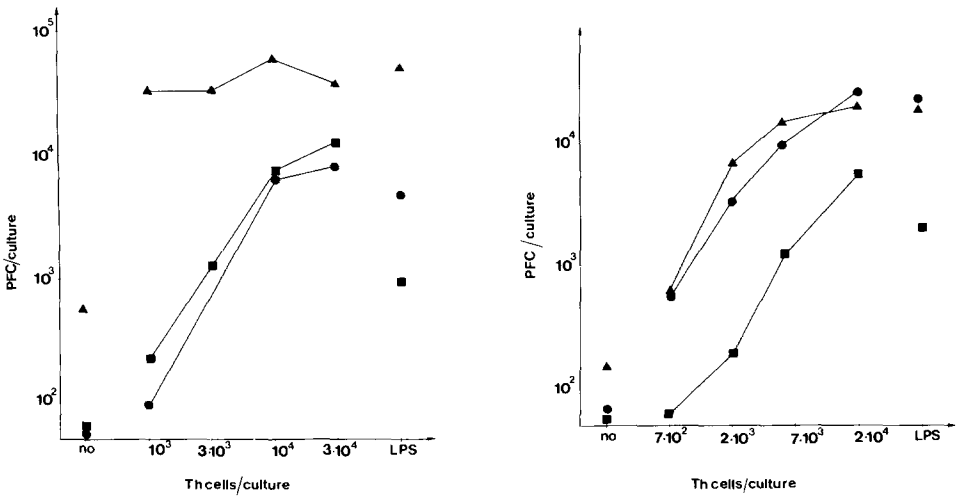

FIG. 7. Day 4 total PFC responses stimulated by the indicated numbers of helper cells or LPS in 4×10^4 T cell-depleted spleen cells (●), T cell-depleted spleen cells passed twice over G10 columns (■), or the latter after reconstitution with 10^4 irradiated, T cell-depleted peritoneal cells (▲). Two independent experiments are shown in (a) and (b) panels.

the effects of macrophage depletion on the PFC response induced by T cells. Fig. 7 presents two independent experiments demonstrating that adherent cell depletion on G10 columns had either no effect (Fig. 7 a) or, while reducing PFC responses, did not abolish them at all (Fig. 7 b). In both experiments, LPS-induced PFC responses are clearly more affected by adherent cell removal, again indicating that B lymphocyte responses to helper cells are relatively macrophage-independent, more so at least than the responses to LPS currently considered highly independent of adherent cells. The quantitative variability in these experiments might be due to variable cellular composition in starting populations of spleen cells or to methodological difficulties introduced by prolonged treatments of the cells with deleterious consequences to cell performance and viability. In both experiments, addition of large numbers of peritoneal cells enhances the responses of depleted cells by a factor of 10, but the same is true for LPS-induced PFC.

We tentatively conclude that primed helper T cells, in the absence of adherent cells, are able to induced differentiation on target B cells, although the presence of macrophages may amplify and optimize these B cell responses. It follows that we ascribe to helper T cells the secretion of factors promoting both B cell growth and maturation.

Discussion

In the antibody response to most antigens, Mϕ are required and thought to perform functions of antigen uptake and presentation to lymphocytes (12). The detailed mechanisms of macrophage function, however, are not clearly understood. The finding of homogeneous cell lines, able to present antigen, may allow a better characterization of the fundamental biochemical events involved (13–16). At the cooperative level, Mϕ-T cell interactions are MHC restricted, as first stressed by Rosenthal and Shevach (8). This interaction, which results in T_H growth, also leads to the production of soluble factor(s) affecting B cell growth and maturation (7, 17–20, and unpublished data). It is not clear, however, whether such B cell-specific factors are of T_H or Mϕ origin. We have used an experimental system previously characterized (1) which amplifies the numbers of specifically interacting lymphocytes in cell populations cooperating in a restricted way. In this system, several points have previously been established (1, 6, 7, 21): (*a*) B cells can be activated in the absence of antigen recognition, as demonstrated before using other conditions of stimulation (22, 23). (*b*) The initiation of T-B cell collaboration requires direct cellular contact to render small, resting B cells reactive to growth factors. (*c*) A single population of helper T cells is sufficient to activate and promote full B cell responses in contrast with T-B collaborative models proposing the existence of two functionally distinct T_H cells (24).

In the present set of experiments, the Mϕ requirements for T cell-induced B cell proliferation and T_H cell growth were studied. It is important to point out that the antigen(s) used in this study is naturally expressed on B cell and Mϕ surfaces, circumventing the requirement for antigen "handling" by Mϕ or B cells (25, 26). Our results on T_H cell growth demonstrate the remarkable failure of B cells to support T_H cell proliferation. This inability, which in this system cannot be due to antigen processing and presentation as the same antigen and restricting elements are expressed on B cell and Mϕ surfaces, emphasizes the unique properties of nonlymphoid adherent

cells in T_H cell activation. This result is in apparent contradiction with other experiments, where a stimulatory effect of B cells is observed with tumor cell lines (13–15) and even with normal lymphocytes (25). On the other hand, a large volume of work has accumulated to indicate that adherent cells are the key elements in driving helper T cells through the mitotic cycle (9, 27–29). The role of macrophages in T cell-induced B cell growth and maturation was also analyzed, and the results lead us to conclude that adherent cells were not an essential element at this level, although a positive effect of Mϕ could readily be demonstrated. In other words, the effector phases of helper cell activity appear to be Mϕ independent. This conclusion may be limited to helper cells that have been extensively primed and many times restimulated in vitro, such as those used here. It can be assumed that such helper cells maintain their "activated" state in the absence of mitotic activity for several days after the last contact with competent stimulator cells and can, therefore, effect helper activity to B lymphocytes in the absence of restimulation. It follows that the present experiments cannot be interpreted to indicate that B lymphocytes, although unable to induce T_H cell growth, are competent to activate effector functions in helper cell populations. It could be argued that our conclusions on the (relative) Mϕ independence of T_H cell-induced B cell growth and maturation are unwarranted, as they are derived from experiments containing irradiated "stimulator" cell populations—to measure helper cell proliferation, with the same populations but nonirradiated—to quantitate B cell proliferation. If the stimulator (or filler effects) of spleen cells were radiation sensitive, then this comparison would be misleading. It has been repeatedly shown, however, that the stimulator function of antigen-presenting cells is radioresistant (2) and even the available reports (13–15, 25) on the stimulatory activity of B cell tumors or nonadherent spleen cells show its resistance to 10,000 and 4,500 rad, respectively. Actually, the novelty of our findings relates not so much to the failure of B lymphocytes to stimulate T_H cell growth (2), but rather to the relative Mϕ independence of B cell growth and maturation, and this conclusion is supported by several other findings. Thus, Mϕ-depleted populations, as shown not only by their failure to stimulate T_H growth after irradiation but also by a 10-fold reduction in LPS-induced responses, do proliferate upon stimulation by helper cells. Unquestionably, the proliferating cells include B lymphocytes, as shown by the development of PFC responses (Fig. 7) and the fact that such populations stimulated with irradiated T_H cells show high levels of proliferation regardless of whether or not they are macrophage depleted (e.g., Figs. 2 and 6). The enhancement of B cell responses after reconstitution of lymphocyte cultures with irradiated peritoneal cells can be explained in three distinct ways: first, it can be the result of "nonspecific" feeder or filler effects, leading to improved cell viability and performance; this possibility is made more plausible by the fact that the effect of Mϕ reconstitution is more marked if helper cells were irradiated and, consequently, less viable. Second, the effector functions of activated T_H cells may rapidly decay in the absence of macrophages; therefore, addition of adherent cells may simply amplify helper activity. Finally, we cannot exclude the production by Mϕ of growth and maturation factors for B cells (17, 30–32), although as shown here, these do not appear essential to B cell responses. There is now strong evidence for a T cell origin of B cell growth factors (33, 34) and a typical maturation factor such as T cell-replacing factor is claimed to be produced by helper T cells as well (35). Furthermore, we have recently isolated a helper clone that,

upon interaction with competent macrophages, produces B cell-specific growth but not maturation factors, indicating that such maturation factors are most likely produced by the helper T cells themselves (S. Pettersson, G. Pobor, A. Bandeira, and A. Coutinho. Manuscript in preparation). In our experiments, we observed a striking Mϕ dependence of LPS responses, in contrast with some published data (36), and in agreement with others (M. Howard and F. Melchers, private communications, and 37). Either the first or the last of the above alternatives can be at the origin of these findings.

In conclusion, a major quantitative difference in macrophage requirements for growth exists between T_H cells and T-dependent B cells. We consider, therefore, that macrophages control cooperative B cell growth indirectly, by promoting the numbers and effector functions of T_H cells.

Summary

Major histocompatibility complex-restricted helper T cell clones against "minor" antigens expressed on B cell and macrophage surfaces, when confronted with appropriate T cell-depleted spleen cells, are induced to proliferation and, in turn, activate "target-responder" B cells to polyclonal growth and maturation. Irradiation of helper cell populations, however, demonstrates that their effector functions (and B lymphocyte responses) are independent of proliferative activity. Adherent cell depletion on Sephadex G10 columns, while completely abrogating helper T cell proliferation, does not abolish helper cell-induced B cell responses, demonstrating a remarkable quantitative difference in macrophage requirements for the growth of these two cell types. Because significant B cell responses are detected upon interaction with primed helper T cells under conditions of extreme macrophage depletion, we conclude that the role of macrophages in T-B cell cooperation is limited to expansion of optimal numbers of helper T lymphocytes. It follows that activated helper cells can autonomously produce all B cell-specific growth and maturation factors mediating cooperative antibody responses. In contrast, the profound reduction of LPS-induced responses upon macrophage depletion suggests accessory cell production of such factors in thymus-independent B cell growth and/or maturation.

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