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Activation and expansion of hapten- and protein-specific T helper cells from nonsensitized mice

CONRAD HAUSER AND STEPHEN I. KATZ*

Dermatology Branch, National Cancer Institute, Bethesda, MD 20892

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ABSTRACT **Hapten- and protein-antigen-specific T helper cells are usually expanded *in vitro* from lymphocytes obtained from sensitized animals. In this paper we report on the primary activation and proliferation *in vitro* of T helper cells from nonsensitized animals by using syngeneic cultured epidermal Langerhans cells as a source of potent antigen-presenting cells. The primary *in vitro* proliferation was blocked with monoclonal antibodies to Ia molecules, to lymphocyte function-associated antigen 1 (LFA-1), and to L3T4. T helper cell populations sensitized *in vitro* to haptens and protein antigens showed hapten- and antigen-specific proliferation when restimulated *in vitro* with spleen cells. Besides its experimental usefulness, *in vitro* generation of syngeneic specific T helper cells may afford possibilities for adoptive immunotherapy.**

The generation of T helper cell populations specific for haptens or soluble protein antigens is currently performed by painting skin with the hapten or by injecting the antigens with adjuvant into skin or muscle of naive animals. T helper cells from such sensitized animals are then expanded *in vitro* by periodic restimulation with hapten or antigen and class II histocompatible antigen-presenting cells. In contrast, allospecific T helper cells can be derived *in vitro* by stimulation with class II histoincompatible antigen-presenting cells without previous *in vivo* sensitization. Cells thereby generated are useful in the study of various aspects of T helper cell biology. This report describes the activation and expansion of hapten-specific and protein-specific T helper cells by *in vitro* sensitization of naive T helper cells, thereby circumventing the many immunomodulatory factors that influence T helper cell generation during *in vivo* sensitization.

When epidermal Langerhans cells are cultured for 1–3 days, their antigen-presenting capacity increases markedly (1), along with an increase in the density of class II major histocompatibility antigen on their cell surfaces (2). Additionally, the cultured Langerhans cells (cLCs) have an increased capacity to form aggregates with lymphocytes (1), which seems to be another critical factor for their augmented antigen-presenting function. The potency of cLCs in various T-cell proliferative assays encouraged us to attempt to generate hapten- and protein-antigen-specific T helper cell lines *in vitro* from nonsensitized mice.

MATERIALS AND METHODS

Preparation of T Helper Cells Depleted of Autoreactivity (Th′ cells). Nylon-wool-filtered C3H or BALB/c lymph node cells, treated with anti-major histocompatibility complex class II monoclonal antibody (mAb) (M5/114.15.2), anti Lyt-2 mAb (53-6.72), mouse anti-rat κ chain mAb (MAR-18.5), and complement (Cedarlane, Hornby, ON), were cultured at 6×10^6 cells per well with nonmodified, 1500-rad (1 rad = 0.01 Gy) irradiated cLCs (3×10^5 cells per well) in 2 ml of culture medium in 24-well culture plates (Costar, Cambridge, MA) for 4 days. The cLCs were obtained from the interphase of a Lympholyte M gradient (Cedarlane) that was overlayered with syngeneic 3-day cultured nonadherent epidermal cells (prepared as described in ref. 2) and spun for 40 min. Twenty to fifty percent of the resulting interphase cells were major histocompatibility complex class II-positive cLCs as assessed by immunofluorescence microscopy and fluorescence-activated cell sorter analysis. The remaining cells were mainly keratinocytes. RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), Fungizone (2.5 μg/ml), Gentamycin (50 μg/ml), 2 mM glutamine (all from GIBCO), 2×10^{-5} M 2-mercaptoethanol, and indomethacin (1 μg/ml) (both from Sigma) was used as culture medium. Indomethacin was used to block keratinocyte-derived prostaglandin E_2, which inhibits most T-cell responses (T. Tsuchida, M. Iijima, and S.I.K., unpublished data). Bromodeoxyuridine (Sigma, 2 μg/ml) was added on days 2 and 3 to the T-cell–cLC culture. For enhancement of the effect of bromodeoxyuridine and light (3), bisbenzimide (Calbiochem, La Jolla, CA) was added 2 hr prior to exposure of the cultures to a fluorescent light source for 60 min at 4°C. Thereafter, the cells were washed and treated with anti-major histocompatibility complex class II and Lyt-2 mAb (53-6.72), anti-interleukin 2 (IL-2) receptor mAbs (3C7 and 7D4), mouse anti-rat κ chain mAb (MAR-18.5), and complement. Viable cells, usually 30–40% of the original T-cell number, were recovered from the interphase of a Lympholyte M gradient, washed three times, and used for sensitizing cultures. These cells are referred to as Th′ cells. In some experiments, Th′ cells were spun over a Percoll gradient (Pharmacia, Uppsala, Sweden). Cells with a density of >65% Percoll (>1.087 g/ml) were collected and washed three times.

Sensitizing Cultures. cLCs were washed twice in Hanks' balanced salt solution (Biofluids) and incubated for 10 min at 37°C in sterile isotonic phosphate-buffered saline (Biofluids) containing 1 mM 2,4,6-trinitrobenzenesulfonic acid (Eastman, Rochester, NY) or fluorescein isothiocyanate (FITC, 200 μg/ml; Sigma). The cells were then washed three times in culture medium and irradiated at 1500 rads. Hapten-modified cLCs (0.5×10^5 cells) were cultured with 5×10^6 Th′ cells in culture medium in 24-well culture plates (Costar) for 5–8 days. Cells were then used for restimulation assays. To determine primary proliferation, Th′ cells prepared as described above were cultured at 10^5 cells per well in 200 μl of culture medium in flat-bottomed 96-well plates (Costar) and various numbers of nonmodified or hapten-modified cLCs. After 5 days of culture [*methyl*-^{3}H]thymidine (Amersham) incorporation was assessed after a pulse (1 μCi per well; 1 Ci

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Abbreviations: cLC, cultured Langerhans cell; Th′ cells, T helper cells depleted of autoreactivity; mAb, monoclonal antibody; TNP, 2,4,6-trinitrophenyl; FITC, fluorescein isothiocyanate; IL-2, interleukin 2.

*To whom reprint requests should be addressed.

= 37 GBq) for the final 16 hr of culture. To sensitize to soluble protein antigens, 5×10^6 Th′ cells were cultured with 10^5 nonmodified 1500-rad irradiated cLCs in the presence of cytochrome *c* (1 mg/ml) (Sigma) or ovalbumin (1 mg/ml) (Sigma) in 2 ml of culture medium in 24-well plates (Costar). After 5 days, recombinant human interleukin 2 (IL-2, 50 units/ml; Cetus, Emeryville, CA) was added. On day 10, the cells were restimulated with antigens (1 mg/ml) and spleen cells (3×10^6 cells per well, irradiated with 3300 rads). IL-2 was added on day 11. At day 20, the cells were used for restimulation assays.

Restimulation Assays. Sensitized T helper cells were treated with anti-major histocompatibility complex class II mAb (M5/114.15.2) and complement. The cells were then spun over a Lympholyte M (Cedarlane) gradient. Washed interphase cells were cultured at $1–2 \times 10^4$ cells per well and various numbers of nonmodified or hapten-modified, 3300-R irradiated spleen cells in 200 μl of culture medium in flat-bottomed 96-well plates (Costar). After 3 days of culture and a [^{3}H]thymidine (Amersham) pulse (1 μCi per well) for the final 12 hr, proliferation was assessed by incorporation of radioactivity into cells.

RESULTS AND DISCUSSION

Preliminary experiments demonstrated that hapten-modified cLCs elicited strong proliferation of Lyt-2-depleted T cells. However, strong—and significantly lower—proliferation was also observed when nonmodified cLCs were used. To diminish this autologous response, Lyt-2-depleted (T helper) cells were precultured with nonmodified cLCs. Activated cells were eliminated with bromodeoxyuridine and light treatment (3) as well as with cytolytic depletion by antibodies to the IL-2 receptor and complement. The remaining T helper cells (defined here as Th′ cells) were not responsive to recombinant IL-2 (data not shown) and showed minimal reactivity to reculture with autologous nonmodified cLCs (Fig. 1*A*). As determined by fluorescence-activated cell sorter analysis, there was no contamination with Lyt-2-bearing cells and these Th′ cells exhibited the same size profile as did freshly prepared T helper cells (data not shown).

Hapten-modified cLCs elicited a strong proliferative response in these Th′ cells (Fig. 1*A*). In addition, IL-2, assessed as CTLL growth-promoting activity, was produced by the Th′ cells that were incubated with hapten-modified cLCs, but not by cells that were incubated with nonmodified cLCs (data not shown). Results were similar in C3H and BALB/c mice. Antibodies to relevant I-A and I-E molecules inhibited the response of Th′ cells to hapten-modified cLCs partially when added separately to the cultures and inhibited the response by 90% when added together (Fig. 1*B*). However, antibodies to irrelevant class II antigens did not block the response. Furthermore, antibodies to the lymphocyte function-associated antigen 1 (7) and L3T4 (8) molecules completely blocked Th′-cell proliferation to hapten-modified cLCs (Fig. 1*C*). Antibodies to Lyt-2 (53-6.72), Thy-1 (30H12,H013.4), Mac-1 (MI/70.15.11.5), NLDC145 (obtained from G. Kraal, Free University Medical Center, Amsterdam), a mAb to a protein expressed on cLCs and other dendritic cells (9), failed to inhibit proliferation. To determine whether the proliferating cells had been activated and thereby were enlarged and less dense, high-density (small resting) Th′ cells were purified by centrifugation through Percoll. These cells also proliferated in response to hapten-modified, but not to nonmodified, cLCs (Fig. 1*D*). Furthermore, when Lyt-2-depleted thymocytes (containing L3T4-bearing cells) were depleted of autoreactivity and purified for high-density cells, proliferation to hapten-modified, but not to nonmodified, cLCs was seen (data not shown). These results indicate that it is unlikely that T helper cells that were recently activated were proliferating in response to hapten-modified cLCs *in vitro*.

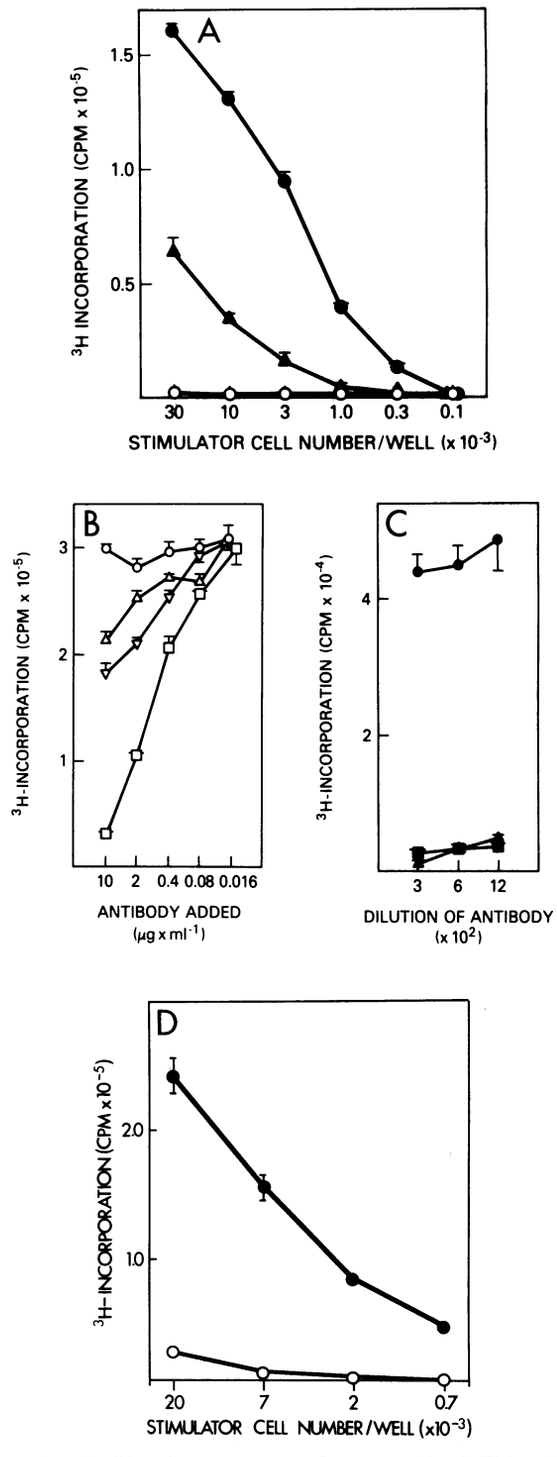

FIG. 1. Proliferative response of nonsensitized Th′ cells to hapten-modified cLCs and its inhibition by antibodies to cell surface molecules. (*A*) Th′ cells (10^5 cells) were cultured with the indicated numbers of nonmodified cLCs (○) or cLCs coupled with 2,4,6-trinitrophenyl (TNP) (●) or with FITC (▲). Proliferation was assessed by [^{3}H]thymidine incorporation. Stimulator cell number corresponds to the number of cLCs used. Background cpm for cultures containing only stimulator or responder cells was <450 cpm. (*B* and *C*) Effect of mAbs to cell surface determinants on the proliferation of Th′ cells to hapten-modified cLCs (10^4 cells per well). C3H (*B*) and BALB/c (*C*) Th′ cells were cultured for 5 days with 10^4 TNP-cLCs in the presence of mAb with specificity for I-A (10.2.16) (△) (4) and I-E^k (14.4.4s) (▽) (5) or a mixture of these two (□), I-A^d (MKD-6) (○) (6), leukocyte function-associated antigen 1 (LFA-1) (FD411.8) (▲) (7), and L3T4 (GK1.5) (■) (8). Control SP-2 ascitic fluid was also used (●). (*D*) BALB/c high-density (>1.087 g/ml) Th′ cells were cultured with either TNP-modified (●) or nonmodified (○) cLCs. Points represent means of triplicate cultures. Error bars indicate 1 SEM.

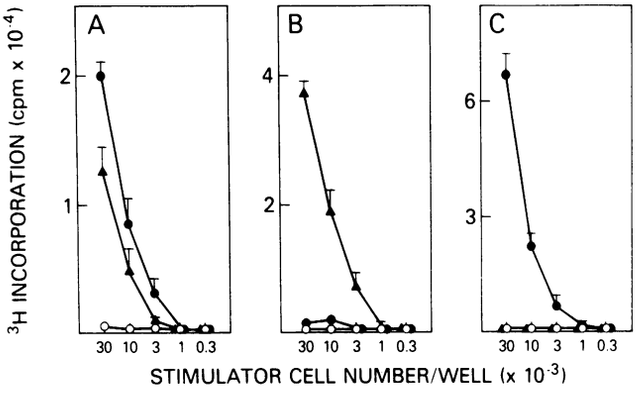

FIG. 2. Proliferation of nonsensitized Th' cells to hapten-modified cLCs is dependent on specific T cells. T helper cells from nonsensitized mice were cultured for 4 days with either nonmodified (*A*) or with TNP (*B*)- or FITC (*C*)-modified cLCs. Proliferating cells were eliminated, and the remaining nonactivated cells were cultured for 5 days with various numbers of nonmodified (○) or TNP (●)- or FITC (▲)-modified cLCs. Proliferation was assessed at day 5 by [^{3}H]thymidine incorporation. Points represent means of triplicate cultures. Error bars indicate 1 SEM. Stimulator cell number corresponds to the number of cLCs used.

To determine whether the observed proliferative response was dependent on the presence of specific T helper cells, we depleted hapten- and autoreactive T helper cells by the methods cited above (bromodeoxyuridine and light; anti-IL-2-receptor antibody and complement) in a first culture. In the second culture, T helper cells responded to cLCs with an irrelevant hapten, but not to cLCs without hapten or to cLCs with the relevant hapten (Fig. 2). This demonstrates that the proliferative response is dependent on the presence of a specific T helper cell and renders the possibility of a non-specific mitogenic effect of hapten-modified cLCs on small resting Th' cells unlikely. Furthermore, the elimination of hapten-specific T helper cells suggests that their activation occurs without the preculture used to eliminate autologously reacting cells, and the elimination of autologously reacting cells suggests that hapten-dependent T helper cell activation is largely independent of an autologous T helper cell reaction. Restimulation of *in vitro* activated T helper cells with spleen cells showed their hapten specificity (Fig. 3). Repeated stimulation of these T helper cells also allowed the establishment of lymphokine-secreting hapten-specific T helper cell lines, which proved to be L3T4 positive and Lyt2 negative (unpublished data).

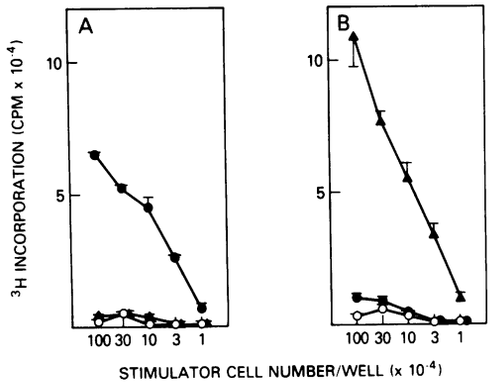

FIG. 3. Proliferative response of *in vitro*-sensitized T helper cells to hapten-modified spleen cells. Th' cells sensitized with either TNP-cLCs (*A*) or with FITC-cLCs (*B*) were treated with anti-class II mAb and complement to eliminate residual cLCs. After centrifugation on Lympholyte M, 10^4 cells at the interface were cultured for 3 days with either nonmodified (○), TNP-modified (●), or FITC-modified (▲) spleen cells. Proliferation was assessed after 3 days by [^{3}H]thymidine incorporation. Points represent means of triplicate cultures. Error bars indicate 1 SEM. Stimulator-cell number corresponds to the number of spleen cells used.

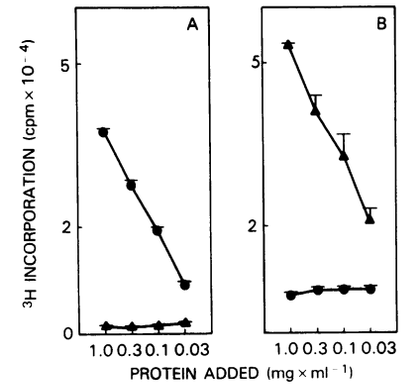

FIG. 4. Proliferative response of *in vitro*-sensitized T helper cells to protein antigens. C3H Th' cells were sensitized to either cytochrome *c* (*A*) or to ovalbumin (*B*). Proliferative responses of *in vitro*-sensitized T helper cells were assessed after 3 days of restimulation with antigen, 3×10^5 spleen cells per well, and 2×10^4 T helper cells per well. Responses are shown for cytochrome *c* (●) and for ovalbumin (▲). Points represent means of triplicate cultures. Error bars indicate 1 SEM.

To investigate whether cLCs may also serve as antigen-presenting cells for the activation of T helper cells with specificity for protein antigen, Th' cells were stimulated with cLCs and chicken ovalbumin or cytochrome *c*. Activated cells, which were expanded by restimulation with antigen, spleen cells, and IL-2, exhibited preferential proliferation to the sensitizing protein when washed free of IL-2 and restimulated with syngeneic spleen cells (Fig. 4). Both protein-specific lines responded to the exogenously added protein in a linear dose–response manner. T helper cell lines with specificity for hen egg lysozyme were also produced (data not shown). All of these T-cell helper lines were L3T4 positive and Lyt2 negative. Although all of these cell lines were established by using cLCs as antigen-presenting cells, it is not known whether cLCs are unique in subserving this function.

Although previous studies have reported (10–13) the primary activation of T helper cells *in vitro*, most have relied on assays of B-cell responses. Expansion of these T helper cells has not been reported. In systems where proliferating T cells have been generated *in vitro* (14, 15), secondary rather than primary responses were investigated. The experiments reported herein show that it is possible to activate T helper cells with desired specificity from nonsensitized animals *in vitro* and that these cells can be expanded *in vitro*. Expansion and administration of effector T cells with specificity for tumor-associated antigens (in mice) or of T cells infiltrating human tumors have been shown to be effective methods for the control of certain tumors (16, 17). Thus, T helper cells generated by *in vitro* sensitization should also be useful in adoptive immunotherapy.

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