Brief Definitive Report

CLONAL EXPANSION OF HUMAN T LYMPHOCYTES INITIATED BY DENDRITIC CELLS

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During an immune response, antigen initiates the clonal expansion of single lymphocytes that are precommitted to recognize that antigen (1). Recognition is mediated by lymphocyte receptors that, in the case of T cells, identify fragments of antigen in association with products of the MHC on the surface of APCs (2). The presenting cells also may provide "accessory" or "feeder" functions that are necessary for the clonal induction of immune responses. A more rigorous analysis of accessory function should emerge by determining the requirements for the production of T cell clones from single precursor cells. It is known that T cell clones can be generated in culture, but that this requires large numbers ($1-3 \times 10^5$) of feeder cells (3–5). The latter are heterogeneous populations usually obtained from mouse spleen or human blood. We have investigated the accessory cell requirements for the growth of individual human T lymphocytes by comparing enriched populations of dendritic cells, monocytes, and B lymphocytes. We find that dendritic cells are active and perhaps essential. Single $CD4^+$ lymphocytes can be cloned with 80% efficiency if 10^3 syngeneic or allogeneic dendritic cells are added, and some clones develop even with one dendritic cell. The efficacy of enriched populations of monocytes or B lymphocytes as feeder cells is 1% or less than that of dendritic cells.

Materials and Methods

Isolation of Human Leukocyte Populations. Populations that were enriched in monocytes, T cells, dendritic cells, or B cells were obtained as described (6) and further purified by panning of the isolated cell fractions. Briefly, monocytes were the low density fraction from Percoll gradients of blood mononuclear cells from which T cells were removed by rosetting with neuraminidase treated sheep red blood cells. Two populations were obtained by allowing the monocytes to attach to plastic after 1–2 d of culture. The adherent cells were almost entirely monocytes and represented 70–80% of the low density fraction. The nonadherent low density cells included some monocytes, lymphocytes, and dendritic cells. T cells were isolated from the high density Percoll fractions by rosetting with neuraminidase-treated SRBC and passed over nylon wool. $CD4^+$ and $CD8^+$ subsets were prepared by coating T cells in OKT4 (50% vol/vol) or OKT8 (25% vol/vol) hybridoma culture medium for 1 h at 5°C, washing, and applying to dishes coated with anti-mouse Ig for two consecutive pannings. The nonadherent cells were >98% depleted of the respective subset as demonstrated by FACS analysis after staining with a combination of PE-anti-CD4 and FITC-anti-CD8 (Becton Dickinson & Co., Mountain View, CA). The remaining, T cell-depleted, high density cells were separated into B cell and dendritic cell-enriched populations by a second reflotation in dense Percoll after 2 d of culture. The low density population contained most of the dendritic cells and was depleted

This work was supported by National Institutes of Health grants AI-24775 and AI-24540. E. Langhoff is a Charles H. Revson and Norman and Rosita Winston fellow.

of residual monocytes by panning on dishes coated with human Ig (6–8), and of residual lymphocytes by panning with mAb to CD22 (B cells; 2 μg/ml G28-7; kindly provided by Dr. E. A. Clarke, University of Washington, Seattle, WA), CD16 (NK cells; 50% vol/vol 3G8 hybridoma medium), and CD5 (T cells; 2 μg/ml Leu-1). The high density B cells from Percoll gradients were handled as described for dendritic cells except that the panning with CD22 mAb was omitted.

Cytofluorography of Cell Isolates. More than 95% of the monocytes stained with the OKM1 anti-CD11b mAb and with 9.3F10 anti-MHC class II, and only 2.5% with the Leu-4 anti-CD3 mAb. The T cells were 95% $CD3^+$ (Leu-4), but only 5% class II (9.3F10), and 1% CD22 (G28-7), as shown by cytofluorography. The dendritic cell fraction consisted of large 15–25-μm cells, most of which extended long processes when examined on a hemocytometer. Few cells in the population stained with mAbs to defined leukocyte subset antigens (3% CD3, 1% CD22, 5% CD11b, 0% CD16), but 82% were rich in class I (W6/32) and II (9.3F10) MHC products. The B cells were <1% $CD3^+$ and $CD11b^+$, and 92% positive for class II and CD22.

In Vitro Cloning of T Cells. T cells, plated at one cell per round-bottomed microtest well (Linbro; Flow Laboratories, Inc., McLean, VA) were supplemented with 1–10,000 (50 μl) of irradiated (1,500 rads) feeder cells. Feeder cells were dendritic cells, monocytes, primary B cells, or EBV-transformed B cell lines (kindly provided by Dr. J. Zabriskie, The Rockefeller University). To each microwell we added 50 μl of T lymphocytes (one cell/well), and 50 μl of growth factor. The latter was conditioned medium made in our laboratory by stimulating blood mononuclear cells (2×10^6 cells/ml) with 20 μg/ml PHA (Difco Laboratories Inc., Detroit, MI) for 48 h in RPMI 1640 supplemented with 10% human serum and antibiotics. Clonal growth was scored from day 5 by phase microscopy (×25), and in some cases by [^{3}H]thymidine incorporation. For the latter, additional lectin-free growth factor (Electro-Nucleonics Inc., Silver Springs, MD) was added to each well at day 10 and the cultures were exposed to 1 μCi/ml [^{3}H]thymidine (6 Ci/mM) for 40 h. A parallel control plate had dendritic cells but no T cells. Significant radioisotope uptake was >1,200 cpm, which was 3 SD above the mean of the control plate.

Results

To test if dendritic cells could induce the clonal expansion of T lymphocytes, we cultured T cells at an average of one cell per well in the presence of a polyclonal mitogen, PHA, and the conditioned medium of PHA-stimulated leukocytes as a source of growth factors (Fig. 1). In two instances, we also plated 0.33 cells/well and observed that the frequency of clones was reduced by 2.5 (expected reduction, 2.4). The clones were apparent within 7 d (Fig. 1, *a* and *b*), beginning as a tight ball and then expanding further during the second week to produce a sizable layer of large lymphoblasts (Fig. 1, *c* and *d*). The yield of clones, scored by inspection at a final magnification of ×25, was similar whether the scoring was done at 7, 10, or 14 d (not shown). The accuracy of this visual assay was certified by adding growth factor and [^{3}H]thymidine at day 10. Every well with a clone by microscopy also incorporated radiolabel (Fig. 1 *e*).

Very few clones developed if either lectin, growth factor, T cells, or dendritic cells were omitted (Table I). Only a small number of clones developed when 1,000 *unirradiated* dendritic cells were cultured without T cells, indicating that the contamination of the dendritic cell population with T cells was negligible. Supplementation with additional IL-2 (10 U/well) did not increase the frequency of positive wells (not shown). The clones could be transferred at day 10–14 from the microwells to larger, 16-mm wells, and expanded with irradiated adherent mononuclear cells, lectin, and growth factors. For the 14 transferred clones that were counted, a yield of $1.6–34 \times 10^6$ (median of 13×10^6) cloned cells was reached in 3 wk with an av-

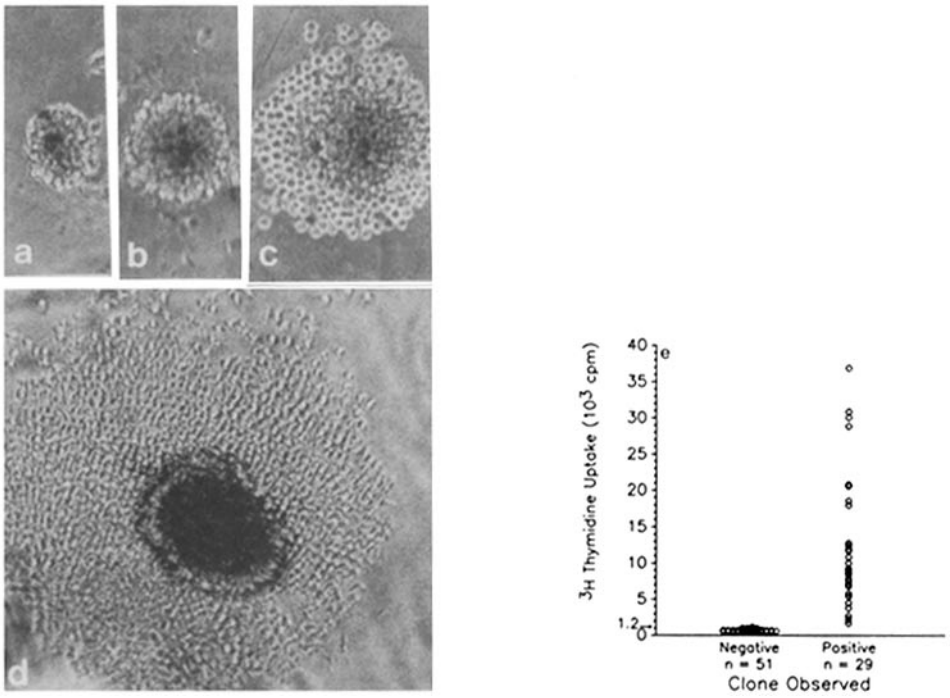

FIGURE 1. Developing T cell clones as observed by inverted phase contrast microscopy. Typical clones were photographed at d7 (*a*, *b*), d10 (*c*), and d14 (*d*) and are shown here at a final magnification of ×125. In *e* it is shown that all clones scored positive by inspection incorporated >1,500 cpm [^{3}H]thymidine, where 1,200 cpm is the mean + 3 SD of the negative control wells.

erage doubling time of 1 d (not shown). Both CD4$^+$ and CD8$^+$ T cells could be expanded with dendritic cells as feeders (Fig. 2). However the efficiency of cloning with CD4$^+$ lymphocytes was 80%, whereas the efficiency with CD8$^+$ cells was 50–60% (not shown). The basis for this difference is currently being explored.

TABLE I

The Requirements for the Production of T Cell Clones as Noted in a Series of Seven Experiments

Number of dendritic cells	Irradiation	T cell	Growth factor	Lectin (PHA)	Yield of clones
1,000	+	−	+	+	0/640
1,000	−	−	+	+	4/200
100	−	−	+	+	0/80
1,000	+	+	+	−	2/80
1,000	+	+	−	+	0/160
0	−	+	+	+	2/560
1,000	+	+	+	+	251/560

Four experiments were with bulk T cells and three experiments were with CD4$^+$ T cells. The numbers indicate clones observed per wells examined. PHA was used as lectin, and lectin-free leukocyte-conditioned medium was used as growth factor (Electro-Nucleonics Inc). Bulk T cells were plated at a frequency of one cell/well. The dendritic cells were irradiated with either 450 or 3,000 rad of ^{137}Cs in different experiments.

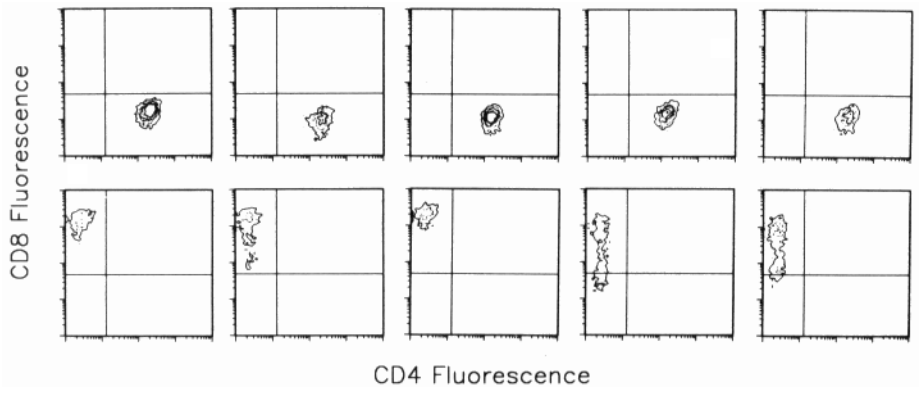

FIGURE 2. Both CD4+ and CD8+ cell clones can be induced by dendritic cells. Shown here are the contents of five individual T cell cloning wells induced from single CD4+ (*top row*) and CD8+ (*bottom row*) precursors and stained directly in the wells with phycoerythrin anti-CD4 and fluorescein anti-CD8.

Dose-response experiments were performed to document the efficiency of cloning with different types of feeder leukocytes. We assumed a maximum of 67% positive wells as dictated by the Poisson distribution when plating is performed at one cell/well. With 1,000 dendritic cells, the efficiency of cloning was 80 ± 12% in four experiments with CD4+ lymphocytes. The yield of clones with dendritic cells varied reproducibly with accessory cell dose, with some clones being detected with only one dendritic cell per well (Fig. 3 *A*). The yield was similar whether the dendritic cells were irradiated with 3,000 rad, 450 rad, or no ^{137}Cs (not shown). Both syngeneic and allogeneic dendritic cells were effective as feeders (Fig. 3 *B*). Highly enriched populations of adherent monocytes had little (Fig. 3 *B*) and sometimes no activity (not shown) as feeders, even at a dose of 10^4 monocytes per well. A plastic nonadherent fraction, which contained monocytes as well as lymphocytes and dendritic cells (6), had more activity than the more enriched adherent monocytes (Fig. 3 *B*). Enriched preparations of primary, blood B cells had 1% of the efficacy of dendritic cells, but EBV-transformed B cell lines were just as active as dendritic cells (Fig. 3 *C*).

Discussion

The capacity of dendritic cells to induce the long-term growth of T lymphocytes reveals a new specialization, probably the ability to induce long-term responsiveness

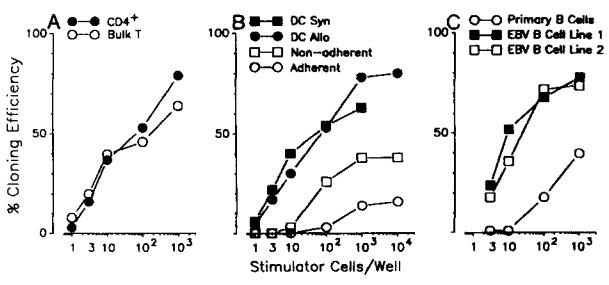

FIGURE 3. Cloning efficiency with different populations of feeder cells. (*A*) Dendritic cells as feeders for bulk and CD4+ T cells. The data for each dose are the median of three to six experiments with CD4+ T cells and three experiments with bulk T cells. (*B*) Comparison of syngeneic and allogeneic dendritic cells vs. monocytes as accessory cells. (*C*) Comparison of primary B cells (see Fig. 1) with two EBV-transformed B cell lines.

to T cell growth factors. Other previously described features of dendritic cells would not seem to explain their efficacy as feeder cells in clonal assays. For example, dendritic cells have very high levels of class II MHC products that are required to present antigens to $CD4^+$ T cells. However, high levels (6 μg/ml) of the 9.3F10 anti-HLA class II mAb, which inhibits dendritic cell function during polyclonal T cell responses in bulk culture (9), did not block function in the cloning assay (not shown). Dendritic cells efficiently bind antigen-reactive $CD4^+$ cells (10, 11). Yet in the cloning assay, a lectin was added that would ensure binding of T cells to many different types of presenting cells particularly monocytes (not shown). Dendritic cells initiate the production of growth factors from bulk cultures of resting lymphocytes (12, 13), but in the cloning assay, exogenous growth factors were added and were required. Given the facts that other class II^+ leukocytes were weak accessory cells (Fig. 3), and that both lectin and exogenous growth factors were needed, we tentatively conclude that dendritic cells are specialized to induce long-term responsiveness to T cell growth factors.

The data (Fig. 3, *B* and *C*) do not distinguish between the possibilities that our monocyte and B cell preparations have a small contamination of dendritic cells, or if these other presenting cells have a reduced but real capacity to act as feeder cells. If the latter were the case, and if the findings could be extended to antigen-specific responses, then one would require at least 1,000 antigen-bearing monocytes or B cells to occasionally induce significant clonal expansion of 1 T cell. Such a situation may not readily occur in situ, especially in the T-dependent areas of lymphoid tissues where monocytes and B cells are relatively scarce (14) but where dendritic cells are localized (15). Therefore, it appears that the initiation of extensive clonal growth of T lymphocytes, even in the presence of exogenous growth factors and lectins or antigens, is tightly controlled such that the trace population of dendritic cells may be essential.

Summary

The accessory cell requirements for cloning T cells in the presence of lectin and T cell growth factors were examined with cells from human peripheral blood. We found that dendritic cells were active and perhaps essential. Single $CD4^+$ lymphocytes could be cloned with 80% efficiency, and $CD8^+$ cells with 50–60% efficiency if 10^3 syngeneic or allogeneic dendritic cells were added. Some T cell clones developed even with one dendritic cell. Monocytes or B lymphocytes from blood were at least 100-fold weaker in supporting clonal growth. These findings suggest a specialized feeder cell requirement, namely dendritic cells, for cloning T lymphocytes from single resting precursors.

We thank Dr. J. Young for helpful advice with cell isolations and Dr. D. Lawless for help with the FACscan studies.

Received for publication 12 September 1988.

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