

LYMPHOKINE PRODUCTION BY MURINE T CELLS  
IN THE MIXED LEUKOCYTE REACTION

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Murine B cell stimulatory factors have been defined using T cell tumors, hybridomas, cell lines, or clones as the source. These factors, such as IL-4, IL-5, and IL-6, each have distinct effects on B cells. The effects of IL-4 on B cells include enhanced expression of MHC class II molecules, costimulation of anti-Ig induced DNA synthesis, induced expression of a low-affinity Fc<sub>ε</sub> receptor, and isotype switching to IgG1 and IgE secretion (1, 2). IL-5 costimulates dextran sulfate-induced B cell proliferation and promotes Ig secretion of preactivated B cells (3). IL-6 stimulates growth and Ig secretion of B cell lines (2). An additional B cell growth factor, referred to as BCGF, maintains the growth of anti-Ig-preactivated B cells (4, 5).

The production of defined B cell stimulatory factors by antigen-activated normal T cells has not been analyzed. T cells activated in an MLR produce T cell growth factors as well as helper factors for antigen-dependent responses (6, 7). In this study, we use bioassays and neutralizing mAbs to demonstrate that IL-2, IL-4, and BCGF are among the lymphokines produced during the primary and secondary MLR.

Materials and Methods

*Mice.* Mice of either sex were used at 8-12 wk of age. BALB/c × DBA/2 F<sub>1</sub> (C<sub>x</sub>D<sub>2</sub>F<sub>1</sub>;H-2<sup>d</sup>), C57BL/6 × DBA<sub>2</sub> F<sub>1</sub> (B6<sub>x</sub>D<sub>2</sub>F<sub>1</sub>;H-2<sup>b<sup>nd</sup></sup>), and C57BL/6 congenic for H-2<sup>k</sup> (B6.H-2<sup>k</sup>) were purchased from Trudeau Institute, Saranac Lake, NY.

*Cells.* Dendritic cells (DC) were prepared from spleen low density cells (8). T cells were nylon wool nonadherent Ia<sup>-</sup>, spleen, and mesenteric lymph node cells, and were enriched for CD4<sup>+</sup> T cells by depleting CD8<sup>+</sup> cells with anti-Lyt-2.2 (HO-2.2, American Type Culture Collection [ATCC] TIB 150) and complement (C, Pel-Freez Biologicals, Rogers, AR) (6, 7). To enrich for CD8<sup>+</sup> cells, CD4<sup>+</sup> cells were depleted with GK1.5 anti-CD4 (ATCC; TIB 207) and C. Splenic B cells were depleted of T cells with mAb and C, and adherent cells by passage through Sephadex G-10. These B cells were further fractionated where indicated by Percoll density gradient centrifugation to yield small B cells of a density >1.08 g/ml. Anti-Ig-activated B lymphoblasts were prepared by stimulating purified high density or total B cells with 5 μg/ml anti-Ig-Sepharose for 2-3 d and are referred to as anti-Ig blasts. Blasts were isolated, free of Sepharose beads, on Ficoll-Hypaque.

*Culture Conditions for the MLR.* Culture medium was RPMI 1640 supplemented with antibiotic/antimycotic, 10% FCS, and 50 μM 2-ME. 5 × 10<sup>5</sup>, 5 × 10<sup>6</sup>, or 5 × 10<sup>7</sup> T cells were seeded in 0.2-ml microcultures, 1-ml macrocultures, or 10-ml cultures (upright in 25-cm<sup>2</sup> T flasks), respectively. Stimulator DC, B cells, or anti-Ig blasts were irradiated (<sup>137</sup>C) and added to a constant number of T cells at the ratios indicated. Clusters of DC and responding

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T lymphocytes were isolated from 48-h macro or bulk cultures by velocity sedimentation on Percoll gradients (6). Clusters that contained the antigen-reactive T cells were recultured at  $2-6 \times 10^5/\text{ml}$  in 1 or 10 ml cultures for an additional 48 h; i.e., 48-96 h of the 1° MLR. Supernatants were harvested at the indicated time points and assayed for lymphokines. Simultaneously, 100- $\mu\text{l}$  aliquots of the resuspended cultures were transferred to microtiter wells and pulsed for 8 h with 1  $\mu\text{Ci}/\text{well}$  [ $^3\text{H}$ ]thymidine.

For the 2° MLR, T blasts were generated by DC-T clusters, isolated at 48 h of the primary MLR, and recultured until 96 h. The blasts were separated from residual clusters (6) and then depleted of residual Ia<sup>+</sup> stimulator cells and contaminating CD4<sup>+</sup> or CD8<sup>+</sup> T cells with mAb and C. T blasts were recultured at  $2-5 \times 10^5/\text{ml}$  with the various irradiated stimulator populations at the indicated ratios.

**Lymphokines.** Recombinant human IL-2 (r-huIL-2) was provided by Dr. S. Rudnick (Biogen, Cambridge, MA). r-muIL-4 was purified on an 11B11 anti-IL-4 (provided by Dr. E. S. Vitetta, Dallas, TX) affinity column from the conditioned medium of an IL-4-transfected HeLa cell line (provided by Dr. T. Honjo, Kyoto, Japan).

**Lymphokine Assays.** IL-4 was detected by two different bioassays using r-muIL-4 as a positive control in both cases. One was the costimulation assay in which IL-4 and anti-Ig together induce [ $^3\text{H}$ ]TdR incorporation in high density or unfractionated murine B cells as described (9). The identity of the active factor was confirmed by inhibiting the response with 2-10  $\mu\text{g}/\text{ml}$  11B11 anti-IL-4 mAb (10). The second bioassay was the growth of the CTLL cell line seeded at  $5 \times 10^3$  cells per well in a final volume of 100  $\mu\text{l}$ . Although CTLL is used for IL-2 bioassays, our cell line also responds to r-muIL-4. The maximal proliferative response of CTLL cells to rIL-4 was much lower than the maximal response to rIL-2, but was specifically inhibited by 11B11 anti-IL-4 and not by S4B6 anti-IL-2.

**IL-2.** IL-2 was detected by its ability to maintain the growth of CTLL cells. Inhibition by the anti-IL-2 mAb S4B6 (11) (provided by Dr. T. Mossman, DNAX, Palo Alto, CA) was used to distinguish the contribution of murine IL-2 and other possible T cell growth factors that might register in this assay. r-huIL-2 was used as a positive control.

**BCGF Assay.** Anti-Ig-activated B lymphoblasts proliferate and secrete Ig in response to reculture in the presence of T cell-derived lymphokines distinct from IL-1, 2, 3, and 4 (4). An ammonium sulfate fraction (50-85%) of conditioned medium from PMA-stimulated EL-4 cells was used as a positive control. We have recently determined that neither purified natural IL-5 (provided by Drs. D. MacKenzie and S. Swain, UCSD, University of California, San Diego, CA) nor rIL-5 (present in the supernatant of IL-5 COS cell transfectants) alone can substitute for EL-4 Sn in this assay (E. Pure, unpublished results). We refer to this activity as BCGF. The response induced by EL-4 Sn or MLR Sn in this assay was inhibited by at most 20% by anti-IL-2 or anti-IL-4 or both, confirming that the factor(s) detected by the growth of preactivated anti-Ig blasts is distinct from IL-2 or IL-4.

## Results and Discussion

**Kinetics of IL-2, IL-4, and BCGF Production by CD4<sup>+</sup> T cells in the MLR.** IL-2 was consistently detected in the media of a 1° MLR as early as 4 h (Fig. 1 A). IL-2 levels increased for the first 48 h and remained high for at least 96 h. S4B6 anti-IL-2 completely inhibited the CTLL response to MLR medium collected during the first 2 d, but the mAb blocked only 40-60% at later time points. Incomplete inhibition of later supernatants could be due to the production of IL-2 in excess of the amount that could be neutralized by the dose of S4B6 used, or another T cell growth factor. Since 11B11 anti-IL-4 had no effect when the 1° MLR Sn was tested on CTLL, and inhibition by 11B11 plus S4B6 was similar to S4B6 alone, the S4B6-resistant activity cannot be attributed to IL-4. T blasts were isolated from the 1° MLR and restimulated with fresh DC in a 2° MLR. Large quantities of IL-2, maximal by 12 h, were detectable in the medium of this 2° MLR (Fig. 1 A, right panel). S4B6 blocked much of this activity and 11B11 enhanced the block.

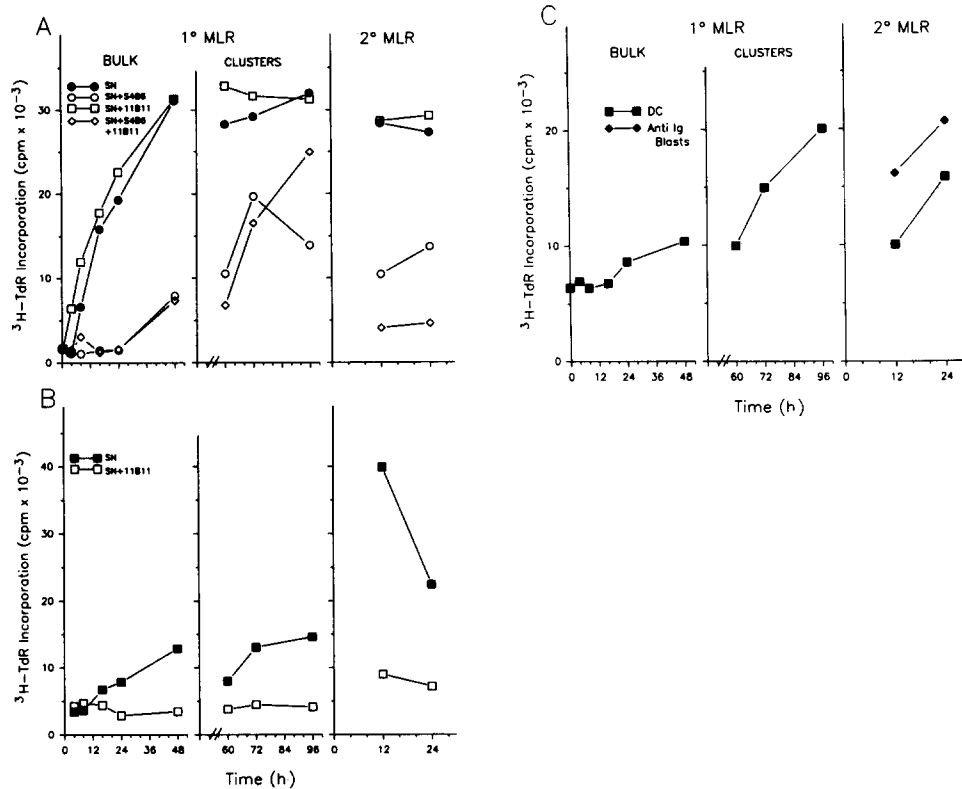


FIGURE 1. IL-2, IL-4, and BCGF production by  $\text{CD4}^+$  T cells during the 1° and 2° MLR.  $5 \times 10^6/\text{ml}$  Cx $\text{D2F1}$   $\text{CD4}^+$  T cells were cultured "in bulk" with  $5 \times 10^4$  B6.H-2k DC in 1 ml for 48 h. Clusters were harvested and recultured at  $5 \times 10^5/\text{ml}$ . T blasts were harvested at 96 h and restimulated at  $5 \times 10^5/\text{ml}$  with  $10^4$  DC or  $5 \times 10^4$  anti-Ig blasts in the 2° MLR. Cell-free supernatants were harvested at the indicated times, and 100  $\mu\text{l}$  aliquots of resuspended cells from the same wells were transferred to microtiter wells to be pulsed with 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]TdR for 8 h to measure proliferation. [ $^3\text{H}$ ]TdR incorporation (cpm) with DC stimulation was: 4 h, 1,687; 8 h, 1,222; 16 h, 1,539; 24 h, 4,002; 48 h, 14,590; 60 h, 22,200; 72 h, 50,690; and 96 h, 96,220 in the 1° MLR. Response to DC in the 2° MLR was at 10 h, 9,132 and 24 h, 22,560; to restimulation with anti-Ig blasts at 10 h, 17,650 and 24 h, 19,940; and unstimulated at 10 h, 3,289 and 24 h, 470. 11B11 anti-IL-4 was added where indicated at 2  $\mu\text{g}/\text{ml}$  and S4B6 anti-IL-2 culture supernatant at 25%. (A) CCTL growth assay of MLR supernatants. (B) Anti-Ig costimulation assay of MLR supernatants. (C) BCGF assay of MLR supernatant on anti-Ig preactivated blasts.

The anti-Ig costimulation assay, in contrast to the CCTL assay which is an insensitive measure of IL-4, detected IL-4 in the 1° MLR Sn. Activity was first noted after the first day and was present for the remainder of the 96 h (Fig. 1 B). The maximal amount of IL-4 detected in the 1° MLR, by comparison with the dose-response curve obtained with r-muIL-4, was 2–10 U/ml in three experiments. The quantity of IL-4 was markedly higher in the 2° MLR Sn (Fig. 1 B), reaching a peak of 100 U/ml by 12 h. The activity of the 1° and 2° MLR media in the anti-Ig IL-4 costimulation assay was completely inhibited by 11B11 anti-IL-4 (Fig. 1 B) but was decreased by at most 20% by S4B6 anti-IL-2 (data not shown).

Finally, MLR supernatants were assayed for BCGF activity on preactivated anti-Ig blasts (Fig. 1 C). BCGF was found in small but detectable quantities by 24 h

in the 1° MLR (Fig. 1 C, left panel). In contrast to IL-4, the BCGF activity increased throughout the next 72 h (Fig. 1 C, center panel). Large quantities of BCGF, comparable with those found late during the 1° MLR, were detected in the conditioned media by 12 h of the 2° MLR (Fig. 1 C, right panel). The response of anti-Ig preactivated blasts was not inhibited at all by 11B11 and at most by 20% in the presence of S4B6 (not shown).

Lymphokine production required T cell stimulation in the MLR. First, the induction of lymphokines was blocked by mAb to the MHC class II antigens expressed on the stimulator population. The mAb did not interfere with the lymphokine bioassays. Second, in all experiments, supernatants from control cultures of stimulator cells alone or fresh responder T cells alone lacked detectable levels of IL-2, IL-4, or BCGF.

The low levels of IL-4 detected in the conditioned medium of the primary MLR may either reflect low levels of production, rapid consumption by the activated T cells that may use IL-4 as a growth factor (12), or the production of factors, for example IFN- $\gamma$ , that inhibit IL-4 activity in the assay.

**Comparison of IL-4 and BCGF Production by CD8<sup>+</sup> and CD4<sup>+</sup> T Blasts.** Inaba et al. (7) have reported that allogeneic DC induced the release of IL-2 by both CD4<sup>+</sup> and CD8<sup>+</sup> lymphocyte subsets. We therefore assayed the conditioned medium from Lyt-2<sup>-</sup> (CD4<sup>+</sup>) or L3T4<sup>-</sup> (CD8<sup>+</sup>) MLRs for IL-4 and BCGF activity (Fig. 2). Conditioned medium from CD4<sup>+</sup> cultures contained IL-4 (Fig. 2 A) and BCGF (Fig. 2 B) by 24 h with maximal levels at 48 h (Fig. 2). The production of IL-4 and BCGF was inhibited by the presence of anti-L3T4 antibody during the MLR (data not shown). In contrast, only low levels of IL-4 and BCGF activity were detected in the conditioned media of the CD8<sup>+</sup> MLR (Fig. 2).

**Efficacy of Allogeneic DC, B Cells, and Anti-Ig Blasts as Stimulators of Lymphokine Production.** DC were the most efficient inducers of IL-2, IL-4, and BCGF in the 1° MLR of CD4<sup>+</sup> T cells. However, anti-Ig blasts also induced high levels of lymphokine production. Both cell types induced T cell proliferation. In contrast, B cells were weak or inactive in stimulating the growth of allogeneic T cells, as well as lym-

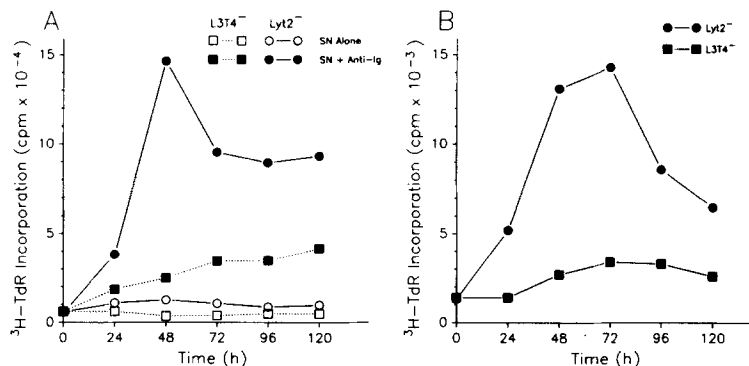


FIGURE 2.  $5 \times 10^6$  CD4<sup>+</sup> (Lyt-2<sup>-</sup>) or CD8<sup>+</sup> (L3T4<sup>-</sup>) CxD2F1 T cells were stimulated with  $5 \times 10^4$  B6xD2F1 DC in 1 ml. The cell-free culture supernatants were harvested at the indicated times and assayed for (A) IL-4 activity in the anti-Ig costimulation assay. (B) BCGF activity on anti-Ig preactivated blasts.

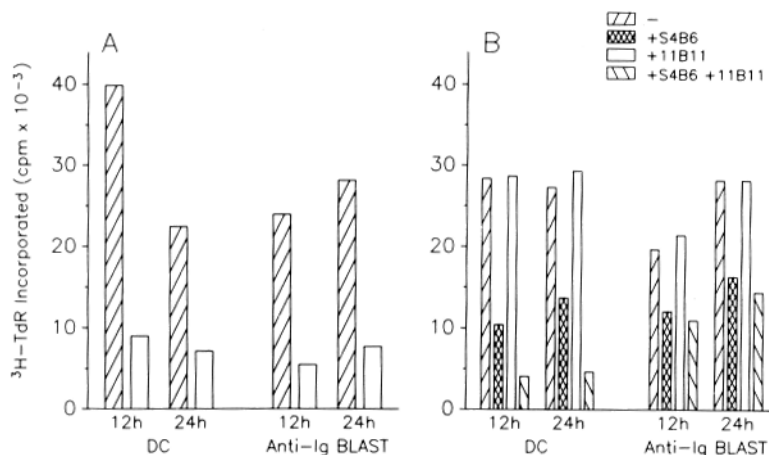


FIGURE 3. Identification of IL-4 and IL-2 in the 2° MLR-conditioned medium. CxD2F1 CD4<sup>+</sup> T blasts were restimulated by B6.h-2k DC or 48-h anti-Ig blasts as in Fig. 1. At 24 h, the medium was taken and assayed for IL-4 in the anti-Ig costimulation assay (A) and in the CTLL assay (B). IL-4 and IL-2 activities were confirmed by comparing the responses in the presence or absence of 2 µg/ml 11B11 anti-IL-4, 25% S4B6 anti-IL-2 culture supernatant, or both.

phokine production (data not shown). The pattern of stimulation in the 2° MLR was different. Small B cells did induce IL-2, IL-4, and BCGF production by allogeneic T blasts, although they were less efficient than anti-Ig blasts or DC (Fig. 3 and data not shown). Consistent with the reported irradiation sensitivity of B cell accessory function (13), treatment with 3,000 rad blocked the capacity of B cells, but not anti-Ig blasts or DC, to induce lymphokine release (data not shown). Interestingly, anti-Ig blasts were reproducibly as, or more, effective than DC at stimulating lymphokine production in the 2° MLR (Fig. 3).

### Summary

Although the production of B cell stimulatory factors by cell lines and hybridomas is well established, production of specific lymphokines by normal T cells in response to antigen stimulation has not been analyzed. We have used bioassays and neutralizing mAbs to demonstrate that IL-2, IL-4, and B cell growth factors (BCGF) are produced during primary and secondary MLRs. IL-2 is detected in the first 12 h of both types of MLR. IL-4 and BCGF appear at 24–48 h in the conditioned medium of the primary MLR, and peak by 12 h in the secondary MLR. The amount of IL-4 in the primary response reaches a level that is 10% of that detected in the secondary. In contrast, BCGF production steadily increases over time in the primary MLR, and maximal production is equivalent to that made in the secondary response. Allogeneic dendritic cells and anti-Ig-activated B blasts both stimulated lymphokine production in the primary MLR, whereas small B cells were weak. In the secondary MLR, all three cell populations stimulated the production of IL-2, IL-4, and BCGF. Therefore, the release of several defined B cell stimulating factors can be detected in the conditioned media of responding primary T lymphocytes.

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