INFECTION AND IMMUNITY, Aug. 1985, p. 298–304
0019-9567/85/080298-07$02.00/0

Vol. 49, No. 2

Dissociation between Interleukin-1 and Interleukin-2 Production in Proliferative Response to Microbial Antigens: Restorative Effect of Exogenous Interleukin-2

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Received 4 February 1985/Accepted 6 May 1985

The relationship between the production of interleukin-1 (IL-1) and interleukin-2 (IL-2) after stimulation of human mononuclear cells within an antigenic extract from *Candida albicans* was analyzed in both responder and nonresponder donors. Culture supernatants from responders contained both IL-1 and IL-2 activity, whereas the supernatants from nonresponders contained only IL-1 and no appreciable IL-2. However, the addition of exogenous IL-2 to nonresponder cultures restored the normal proliferative response. Similar observations were made when cells from mice infected intravenously with high doses of *Mycobacterium bovis* BCG were cultured; these cells showed a marked impairment of the proliferative response to purified protein derivative. Spleen cells from BCG-induced unresponsive mice failed to produce IL-2 despite the fact that normal IL-1 activity was present in the culture. Again, the addition of exogenous IL-2 fully reversed the proliferative unresponsiveness. Thus, the presence of IL-1 does not necessarily induce production of IL-2, and the proliferative unresponsiveness is therefore due to a primary lack of IL-2.

It is widely accepted that T-cell activation requires both the presence of antigen with major histocompatibility complex products and the presence of antigen-nonspecific, genetically unrestricted, soluble factors generally called interleukins. Interleukin-1 (IL-1) is a monocyte-derived factor able to induce the differentiation of T cells and the production of interleukin-2 (IL-2) from antigen-activated T cells (10, 14). IL-2 binds to a specific receptor and allows T cells to pass from the early G_{1a} phase to the late G_{1b} phase. This phase transition is a critical event for the cell cycle, as assessed also by the incorporation of radioactive thymidine (1). In fact, a lack of IL-2 production blocks the cell cycle and DNA synthesis (2).

In the last few years, several pieces of evidence found with both humans and experimental animals have shown that antigen-specific unresponsiveness is accompanied by a failure of T cells to release IL-2. For example, peripheral blood mononuclear cells (PBMC) from lepromatous patients fail to proliferate when stimulated in vitro with *Mycobacterium leprae* antigen, but the cells are fully reactive when cultured in presence of exogenous IL-2 (7). Furthermore, we have recently reported that lack of IL-2 production is responsible for *Mycobacterium bovis*-induced T-cell unresponsiveness in mice (3, 4). The question that arises from these observations is whether the lack of IL-2 production is a primary and IL-1-independent event or whether it is secondary to defective production of IL-1.

This paper analyzes the relationship between the production of IL-1 and IL-2 by using two distinct models, namely, (i) the response of human mononuclear cells either to purified protein derivative (PPD) or to an antigenic extract from *Candida albicans* (15), and (ii) the specific reactivity of spleen cells from *M. bovis* BCG-injected mice to PPD. The results show that in both models the proliferative unresponsiveness is due to a primary lack of IL-2 production and not to a failure of monocytes to release IL-1. The presence of IL-1 does not necessarily induce the production of IL-2 and cell proliferation.

MATERIALS AND METHODS

Microbial antigens. A polysaccharide fraction (MPPS) obtained from *Candida albicans* (15) and PPD from *Mycobacterium tuberculosis* (Statens Seruminstitut, Copenhagen, Denmark) were used as microbial antigens.

Infection of mice. CBA mice, bred locally, were injected either intravenously (i.v.) with 3×10^7 viable *M. bovis* BCG cells (kindly provided by Glaxo, Middlesex, United Kingdom) or intradermally (i.d.) into the right footpad with 5×10^6 BCG cells.

Human and murine cell preparation. PBMC from healthy donors were isolated on Ficoll-Hypaque gradients and suspended in RPMI 1640 medium (Flow Laboratories, Inc., McLean, Va.) supplemented with penicillin, streptomycin, glutamine, and 10% inactivated antologous human serum (culture medium) (15). Primed lymphocytes were prepared by culturing the cells (5×10^5/ml) for 4 days in the presence and absence of 10 μg of MPPS per ml (18). Spleen and popliteal lymph node cells were taken from mice 14 days after i.v. or i.d. injection of BCG and suspended in complete RPMI 1640 medium supplemented with 10% inactivated fetal calf serum (3).

Preparation of IL-1- and IL-2-containing supernatants. Human PBMC and murine spleen and lymph node cells were suspended in culture medium at a concentration of 5×10^5 PBMC per ml and 3×10^6 spleen and lymph node cells per ml. The cell cultures were incubated at 37°C in a CO_2 incubator, and the supernatants were collected after different periods of incubation, filtered through a 45-μm Gelman filter (Gelman Sciences, Inc., Bonn, Federal Republic of Germany), and stored at −20°C until used.

IL-1 assay. A thymocyte proliferative assay was used for

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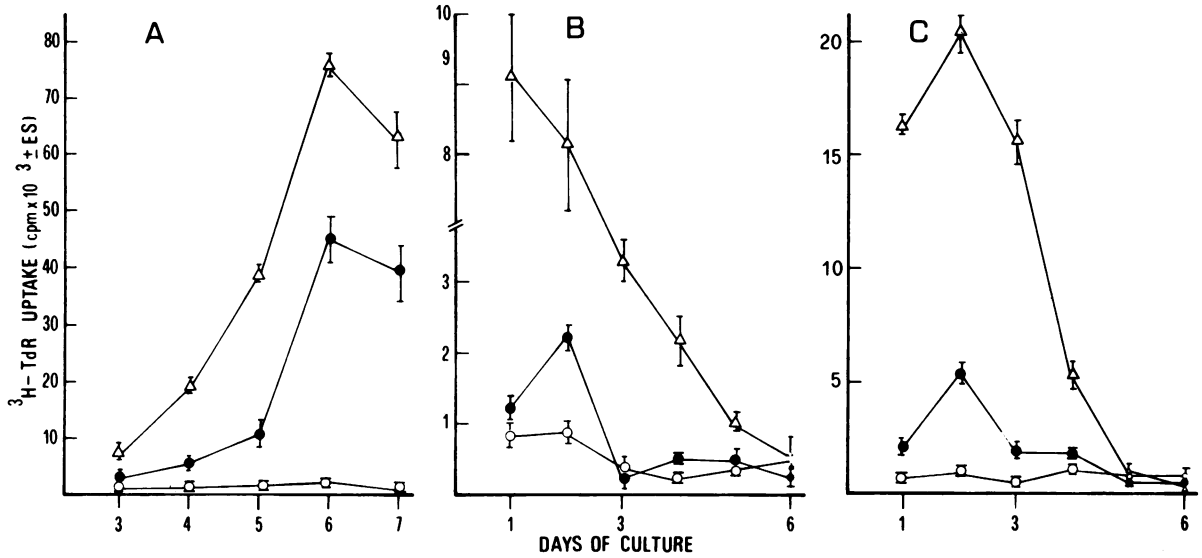

FIG. 1. DNA synthesis (A) and IL-1 (B) and IL-2 (C) activity of PBMC and of supernatants from MPPS-responder healthy donors. (A) [^{3}H]TdR uptake of PBMC cultured alone (○) or in the presence of MPPS (●) or PPD (△). (B) [^{3}H]TdR uptake of mouse thymocytes stimulated with a suboptimal dose of phytohemagglutinin and cultured for 72 h in the presence of supernatants (1/4) from PBMC cultured alone or with MPPS or PPD. (C) [^{3}H]TdR uptake of CTLL cells in the presence of supernatants (1/2) from PBMC cultured alone or with MPPS or PPD. ES, Standard error.

IL-1 (13, 14). Thymus cells from 5- to 7-week-old C3H/HeJ mice, suspended in culture medium at a concentration of 15 × 10^6 cells per ml, were cultured for 3 days in triplicate 96-well microtiter plates in the presence of 0.1 ml of serial dilutions of different IL-1-containing supernatants and 1 μg of phytohemagglutinin (Wellcome Research Laboratories, Beckenham, England). At 6 h before harvest, 0.5 μCi of [^{3}H]thymidine ([^{3}H]TdR) was added.

IL-2 assay. Supernatants from human PBMC cultures and from spleen and lymph node cell cultures from BCG-infected mice were tested for IL-2 activity in a standard microassay by using an IL-2-dependent T-cell line (CTLL) and evaluating the [^{3}H]TdR incorporation (0.5 μCi added) for 10^4 cells in 0.2 ml of medium after 20 h of culture (18).

Exogenous IL-2 preparations. Exogenous IL-2 was prepared by culturing the MLA-144 gibbon T-cell line in serum-free RPMI 1640 as described by Rabin et al. (18). The dilution yielding 50% of the maximum CTLL [^{3}H]TdR incorporation was determined and arbitrarily assigned a value of 1 U/ml. Under our conditions 1 U/ml corresponded to a 1:20 dilution of gibbon IL-2 preparation, and 5 U/ml was used in the reconstitution experiments.

Recombinant human IL-2 (rIL-2) purified to homogeneity from induced *Escherichia coli* K514 cells harboring the plasmid pTrp-Hil201 was kindly provided by Biogen (5), and 20 U/ml was used in the reconstitution experiments.

Lymphocyte proliferation assay. Unprimed PBMC and spleen cells from BCG-infected mice were set up in triplicate in 96-well microtiter plates (Becton Dickinson Labware, Oxnard, Calif.), with each well receiving 10^5 cells in 0.1 ml of culture medium. MPPS (10 μg) or PPD (10 to 20 μg) in 0.1 ml of medium was added. Plates were incubated for different times at 37°C in a CO_2 incubator. At 20 h before harvest, the cultures were pulsed with [^{3}H]TdR (0.5 μCi), and the radioactivity was evaluated in a beta counter and expressed as counts per minute ± standard error. The lymphocyte proliferation assay was used to distinguish responder from nonresponder groups. If the stimulation index, defined as counts per minute from stimulated cells/from unstimulated cells, was less than 4, the donor was considered a nonresponder to the antigen.

IL-2-dependent stimulation assay. PBMC primed with antigen for different periods of culture and spleen cells from BCG-infected mice were harvested, washed, and resuspended in fresh culture medium. The cells (10^5 per well) were cultivated in triplicate for an additional 3 days in the presence and absence of exogenous IL-2. [^{3}H]TdR was added for the last 20 h.

Control PBMC (cells maintained in culture for different times in the absence of antigen) gave a significant proliferative response when rIL-2 was added. However, the [^{3}H]TdR incorporation of primed PBMC was always significantly greater than that of control PBMC.

Analysis of Tac antigen by immunofluorescence. An indirect immunofluorescence assay was used for analysis of Tac antigen. PBMC (10^6) were incubated for 40 min at 4°C with 100 μl of appropriate dilutions of monoclonal antibody to Tac antigen (kindly provided by T. A. Waldmann) (11). After incubation, the cells were washed three times and then incubated with 25 μl of a 1:25 dilution of fluorescein-labeled, affinity-purified antibody to mouse anti-goat IgG (γ-chain) (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Md.). After being washed, the cells were brought up to 20 μl and analyzed by fluorescence microscopy (Orthoplan; Leitz).

Statistical analysis. Data are expressed as mean ± standard error. The double-tailed Student *t* test was used.

RESULTS

DNA synthesis and IL-1 and IL-2 production after stimulation of PBMC with PPD and MPPS. PBMC from healthy donors were cultured in the presence or absence of PPD and MPPS. The DNA synthesis and the production of IL-1 and IL-2 were assessed at different times of culture. PBMC incorporated the maximum amount of [^{3}H]TdR 6 days after

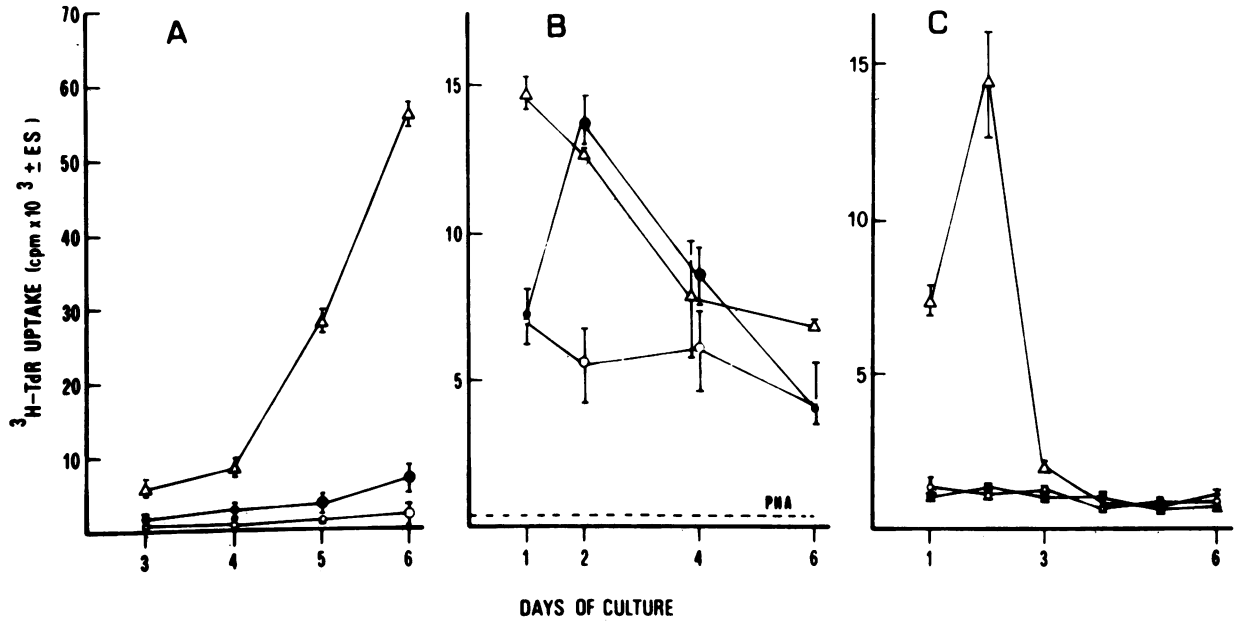

FIG. 2. DNA synthesis (A) and IL-1 (B) and IL-2 (C) activity of PBMC and supernatants from MPPS-nonresponder healthy donors. For details, see the legend to Fig. 1.

antigen stimulation (stimulation index = 19) (Fig. 1A). The IL-1 activity of supernatants collected from PPD- or MPPS-stimulated cultures is shown in Fig. 1B; the maximum amount of IL-1 was observed at day 1 of culture in the PPD system and at day 2 in the MPPS-stimulated cultures.

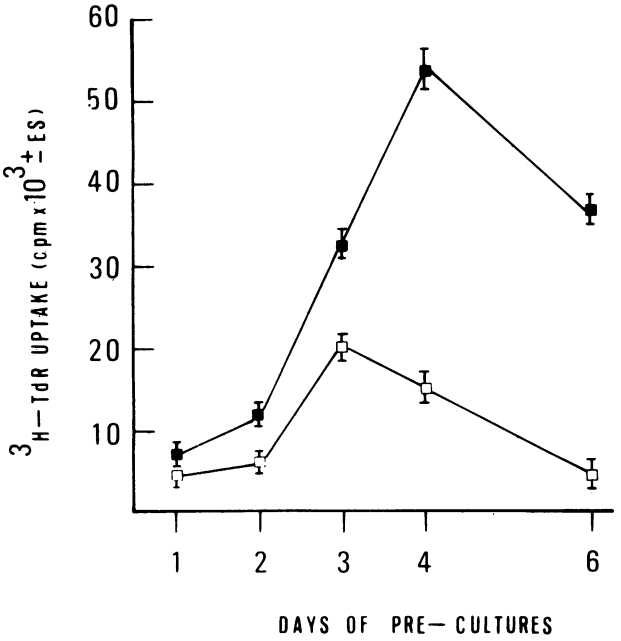

FIG. 3. Effect of pure human rIL-2 on proliferative response of MPPS-precultured lymphocytes. PBMC precultured for different days were maintained for two more days in the presence (■) or absence (□) of rIL-2 (20 U/ml). [^{3}H]TdR incorporation after 20 h of pulse is shown. The proliferation of PBMC precultured for 4 days in the absence of MPPS was 1,522 ± 439 cpm without rIL-2 and 12,998 ± 500 cpm with rIL-2. ES, standard error.

However, the IL-1 levels are not interpretable due to the presence of IL-2. In fact, IL-2 activity was present in 48-h culture supernatants in both the PPD and MPPS systems (Fig. 1C).

A similar protocol was used to culture PBMC from other donors selected as nonresponders to MPPS (stimulation index < 4). DNA synthesis was inpaired in cultures stimulated with MPPS, whereas high levels of [^{3}H]TdR incorporation were observed in the same cultures stimulated with PPD (Fig. 2A). In contrast, monocytes from either PPD- or MPPS-stimulated cultures produced similar levels of IL-1 (Fig. 2B). When IL-2 activity was assessed, PPD-stimulated culture supernatants supported DNA synthesis by CTLL cells, whereas MPPS-stimulated culture supernatants lacked any detectable IL-2 activity (Fig. 2C). These results clearly indicate that IL-1 does not necessarily induce the production of IL-2 in the MPPS response.

A possible criticism of these results is that the thymocyte assay does not distinguish between IL-1 and IL-2. Supernatant taken 2 days after stimulation of PBMC from nonresponder donors was strongly positive in the thymocyte assay but negative in the IL-2 assay (Fig. 2B and C), implying that at least in the case of MPPS the thymocyte assay was measuring IL-1. In contrast, 2 days after stimulation with PPD both the thymocyte and IL-2 assays were positive, and in this situation it is not possible to be certain whether IL-1 or IL-2 is being measured by the thymocyte assay.

Effect of exogenous IL-2 on the restoration of proliferative response to MPPS. We have previously demonstrated that MPPS-stimulated lymphocytes are unable to respond to a second in vitro challenge with the same antigen. However, the proliferative responses of these cells can be restored by the addition of either mitomycin-treated, fresh, non-antigen-stimulated autologous lymphocytes or soluble factors from MPPS-stimulated lymphocytes (17).

The following experiments were performed to investigate the effect of adding exogenous IL-2 to MPPS-stimulated

TABLE 1. [^{3}H]TdR incorporation and Tac expression of PBMC in the absence or presence of rIL-2

rIL-2	Responder				Nonresponder			
	Day 2		Day 4		Day 2		Day 4	
	cpm[a] ± SE	% Tac[b]	cpm ± SE	% Tac	cpm ± SE	% Tac	cpm ± SE	% Tac
−	14,750 ± 1,005	<3	28,600 ± 693	22	1,332 ± 115	<3	2,579 ± 195	13
+	21,435 ± 605	ND[c]	45,038 ± 2,114	ND	6,814 ± 389	ND	21,826 ± 600	ND

[a] [^{3}H]TdR incorporation was evaluated in PBMC stimulated with MPPS for 2 or 4 days and cultured for an additional 48 h in the presence or absence of the optimal dose of rIL-2 (20 U/ml).

[b] The percentage of Tac$^+$ cells on PBMC stimulated with MPPS for 2 or 4 days was obtained by using an indirect immunofluorescence assay. As a positive control, PBMC stimulated with phytohemagglutinin (0.5 μg/ml) gave 37% at day 2 and 26% at day 4.

[c] ND, Not done.

cultures. Figure 3 shows a kinetic experiment in which an optimal dose of pure human rIL-2 (20 U/ml) was added at different times of MPPS stimulation. As can be seen, the most enhancing effect of IL-2 was at day 4 after MPPS stimulation. In a similar experiment, PBMC from both responders and nonresponders were used (Table 1). In this experiment the percentage of cells expressing the receptor for IL-2 (Tac$^+$ cells) also was evaluated. The results confirmed that the effect of exogenous rIL-2 reaches a peak at day 4 after MPPS stimulation, due to an increase in the expression of Tac antigen at this time when compared with day 2. Finally, Fig. 4 illustrates the effect of IL-2 on three responders and nonresponders to MPPS, showing the [^{3}H]TdR uptake of cells from responder donors stimulated with MPPS in the absence and presence of gibbon IL-2 or rIL-2 and the cell proliferation of three nonresponders to MPPS. When gibbon IL-2 or rIL-2 was added to the nonresponder PBMC, a significant increase in [^{3}H]TdR incorporation was observed.

DNA synthesis and IL-1 and IL-2 production after stimulation of spleen and lymph node cells from BCG-infected mice with PPD. The finding that T-cell unresponsivenes is due, at least in part, to a failure of T cells to produce IL-2 has been previously described for BCG-infected mice (3, 4). In the present experiments we investigated the production of IL-1 and the relationship between IL-1 and IL-2 in both responder and nonresponder mice.

Lymph node cells from PPD-responder animals (i.e., mice infected i.d. in the footpad with low doses of BCG) and spleen cells from PPD-nonresponder donors (i.e., mice in-

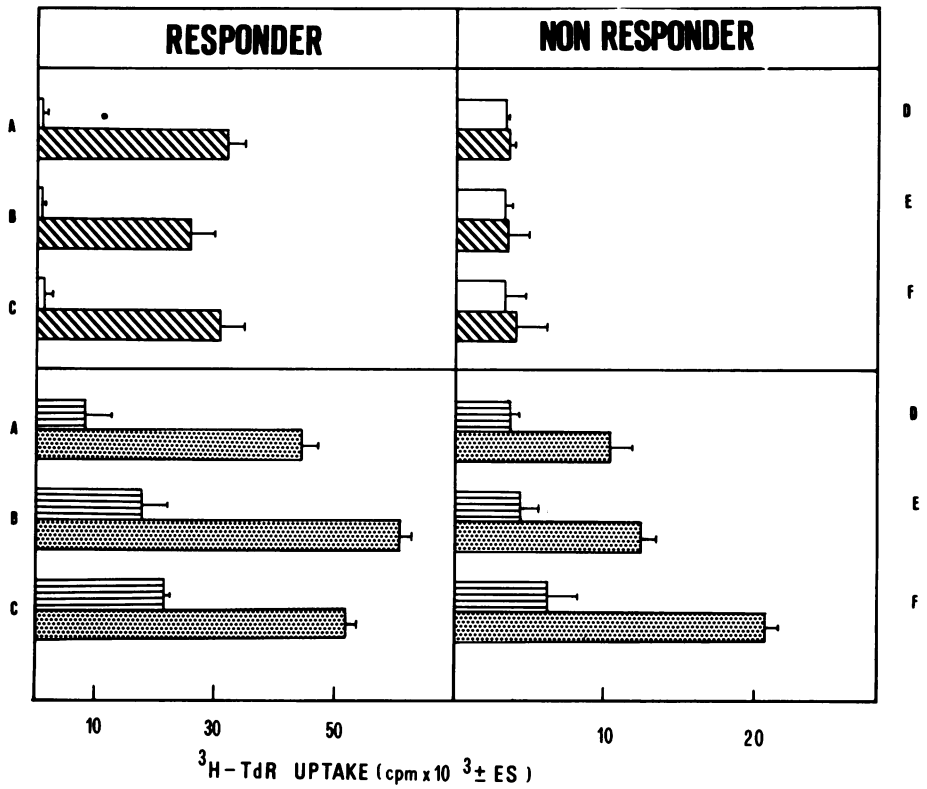

FIG. 4. Ability of exogenous IL-2 to restore the proliferation of PBMC from MPPS-nonresponder donors. The upper panels show the DNA synthesis after 6 days of culture of PBMC from three responders (A, B, and C) and three nonresponders in the absence (▭) or (D, E, and F) presence (▭) of MPPS. The lower panels show the DNA synthesis of 4-day MPPS-activated PBMC (from the same MPPS responders and nonresponders) cultured for 2 days in the absence (▬) or presence (▩) of 5 U of gibbon IL-2 per ml (A, B, D, and E) or 20 U of rIL-2 per ml (C and F). ES, standard error.

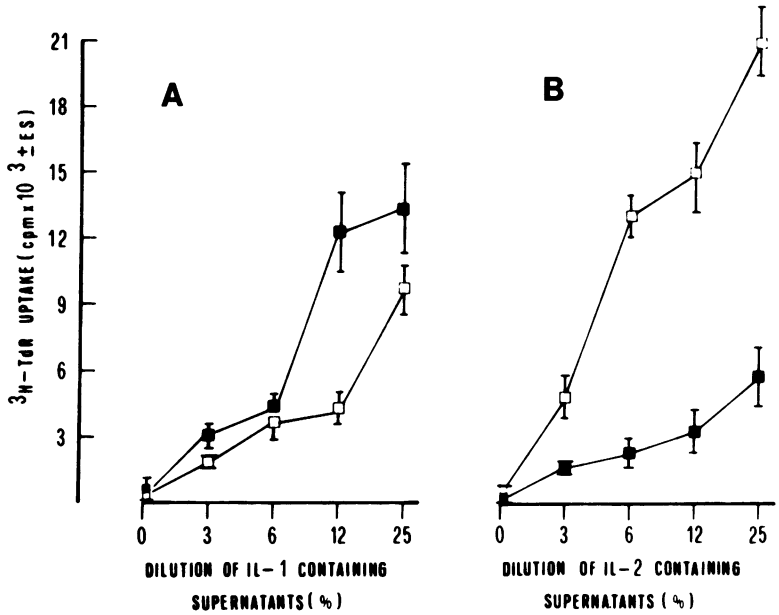

FIG. 5. IL-1 (A) and IL-2 (B) activity of culture supernatants from mice infected i.v. or i.d. with BCG. (A) [^{3}H]TdR uptake of thymocytes in the presence of phytohemagglutinin (1 μg/ml) and serial dilutions of supernatants of spleen cell cultures from i.v.-infected mice (■) or supernatants of lymph node cell cultures from i.d.-infected mice (□). (B) [^{3}H]TdR uptake of CTLL cells in the presence of serial dilutions of the same supernatants. ES, standard error.

fected i.v. with high doses of BCG) produced similar amounts of IL-1 in the culture supernatants (Fig. 5A). In contrast, when IL-2 activity of the culture supernatants was assessed, only supernatants from i.d.-infected mice supported the growth of an IL-2-dependent CTLL line, whereas supernatants from nonresponder animals infected i.v. with BCG failed to do so (Fig. 5B). This difference between the thymocyte and IL-2 assays implies that the thymocyte assay was measuring IL-1, as in the case of PBMC nonresponders to MPPS.

The effect of exogenous IL-2 on lymphocyte proliferation was then investigated. Figure 6 shows the PPD-induced DNA synthesis of spleen cells from responder and nonresponder mice in the presence or absence of gibbon IL-2. Spleen cells from i.v.-infected animals failed to proliferate when stimulated with PPD. However, the addition of exogenous IL-2 fully reversed this unresponsiveness, corroborating our previous observations that the basis of BCG-induced unresponsiveness is a deficiency in the production of IL-2 rather than a lack of reactive T cells.

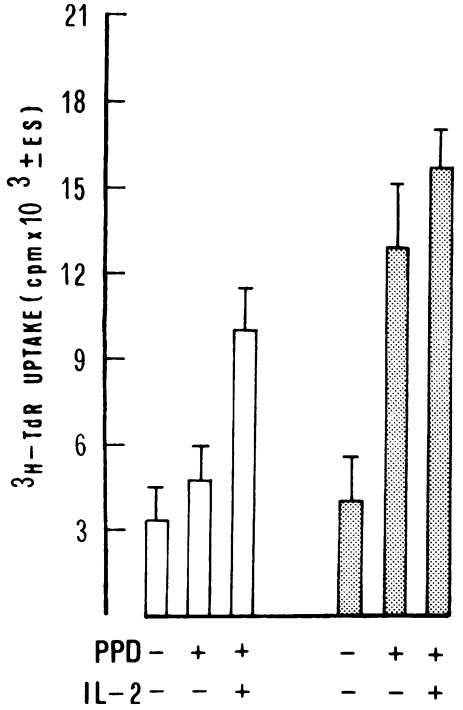

FIG. 6. DNA synthesis of spleen cells from i.v.-infected (▭) and i.d.-infected (▩) mice in the presence or absence of PPD and gibbon IL-2.

DISCUSSION

We have previously reported that both murine and human macrophages release IL-1 when stimulated with MPPS and PPD (13). This paper analyzes the relationship between IL-1 production and the antigen-induced lymphocyte proliferation in responders and nonresponders and studies the restorative effect of exogenous IL-2 preparations.

The lack of the proliferative response of human lymphocytes to *Candida* antigen and of lymphocytes from BCG-infected mice to PPD suggests three conclusions: (i) the nonresponder status is due to a lack of IL-2 production and not to a failure of monocytes to release IL-1; (ii) the presence in the culture of IL-1 does not necessarily induce IL-2 production; and (iii) antigen-reactive T cells from nonresponder humans or animals are present in culture and express IL-2 receptors, as shown by their ability to proliferate when exposed to exogenous IL-2 and by the presence of Tac antigen on PBMC.

It is known that the inability to produce adequate amounts

of IL-2 is implicated in several types of T-cell defects in both humans and mice. For instance, lepromatous leprosy patients fail to respond in vitro to specific antigen and fail to produce IL-2; however, this lack of proliferative response can be reversed by adding exogenous IL-2 (7). Furthermore, both nude and aged mice are defective in production of IL-2, but their cells are fully responsive to exogenous IL-2 (19, 20). However, little information is available on the production of IL-1 in these nonresponder situations. In one study, Linker-Istraeli et al. reported a concomitant defective production of IL-1 and IL-2 in patients with systemic lupus erythematosus, and the impaired IL-2 production was not restored to normal by addition of exogenous IL-1 (12). Hoffenbach et al. reported a decrease in IL-2 activity in supernatants of Conconavalin A-activated spleen cells from C57BL/6 mice infected with *Mycobacterium lepraemurium*, despite the fact that IL-1 production was never affected (8, 9). Identical data have been reported for *Trypanosoma cruzi*-infected mice, which developed a deficit in IL-2 production with normal or enhanced levels of IL-1 (6). In contrast, Watson et al. have recently reported defective production of IL-1 by PBMC from some patients with lepromatous leprosy (21).

In our experiments, we observed situations in which IL-1 is normally present in the culture but the T cells nevertheless fail to release IL-2. Furthermore, the addition of exogenous IL-2 (either gibbon MLA-144 supernatant or pure human rIL-2), but not IL-1 (unpublished data), reverses T-cell unresponsiveness, suggesting that IL-2 receptors are expressed normally on T cells. This has been formally demonstrated by evaluating the expression of Tac antigen on MPPS-stimulated PBMC.

It is known that immunological unresponsiveness can be explained by at least three hypotheses, depending on the experimental model: (i) failure of macrophages to give signal 1 (immunogenic complex between Ia molecules and nominal antigen) or signal 2 (lymphocyte-activating factors, such as IL-1) for T-cell activation; (ii) absence of T-cell clones specific for certain antigenic determinants (i.e., hole in the T-cell repertoire); and (iii) preferential induction of suppressor cells. Our results show that macrophages from both responder and nonresponder cultures are fully able to release IL-1. Furthermore, exogenous IL-2 reverses T-cell unresponsiveness in both models, showing that antigen-reactive T cells are present in the culture. These observations exclude the first two hypotheses as the basis for the immunological unresponsiveness in our models.

We have previously reported that suppressor T cells are actively involved in the regulation of the proliferative response to *C. albicans* in humans (16) and to PPD in BCG-infected mice (4). The present results favor the hypothesis of suppressor cell activation as the basis of unresponsiveness. Indeed, we have recently reported that the interleukin pathway is the target of suppressor cell activity in the MPPS (G. Lombardi, D. Vismara, E. Piccolella, V. Colizzi, and G. L. Asherson, Clin. Exp. Immunol., in press) and BCG (3, 4) systems. Studies on the role of IL-1 in the proliferative response and on its regulation by T-suppressor cells are in progress in our laboratories.

ACKNOWLEDGMENTS

We thank R. Morelli for preparation of MPPS antigen and M. Wirz and O. Pugliese for immunofluorescence analysis with Tac antibodies kindly provided by T. A. Waldmann.

This work was supported by Italian National Research Council (CNR) grants 84.00683 ("Progetto Finalizzato Controllo Malattie da Infezioni") and 84.02029.52 (CNR-Medical Research Council Joint Project).

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