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Patterns of IL-2 production and utilization in mice heavily infected with *Mycobacterium bovis* BCG reflect the phase of protective immunity being expressed

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SUMMARY

The results shown here demonstrate that in mice heavily infected with *Mycobacterium bovis* BCG Pasteur, mitogen-induced levels of interleukin-2 (IL-2) correlate temporally with the state of immunity that is being expressed in the animal during the course of the infection. Active immunity, which is conferred by populations of both $CD4^+$ (L3T4) and $CD8^+$ (Lyt-2) T lymphocytes, and memory immunity, which is mediated by a population of $CD4^+$ T lymphocytes, were identified and distinguished in terms of their sensitivity to cyclophosphamide therapy, their ability to passively transfer specific resistance to infection with virulent *Mycobacterium tuberculosis*, and their capacity to produce and/or absorb IL-2. In this regard, concanavalin A (Con A)-stimulated $L3T4^+$ and $Lyt\text{-}2^+$-enriched splenocytes exhibited an apparent depression in measurable levels of IL-2 when harvested during the first 40 days of the infection, which could be explained by the subsequent observation that these T cells were capable of rapidly absorbing a known quantity of recombinant IL-2 *in vitro*. Detectable levels of IL-2 in these mitogen-stimulated supernatants began to rise after Day 25, which was temporally associated with a gradual shift from active immunity, to immunity mediated by cyclophosphamide-resistant memory T cells, which did not absorb IL-2 *in vitro*. These data indicate that fluctuations in apparent IL-2 production reflect changes in the type of immunity being expressed, rather than than some form of defect in IL-2 production.

INTRODUCTION

Acquired cellular immunity to intracellular bacterial infections, such as *Mycobacterium bovis* BCG, is a complex event involving the interaction of various populations of T cells and macrophages (Mackaness, 1964, 1969; North, 1973; Orme, 1987). One of the controlling elements in these interactions is the production of soluble interleukins (David, 1973; Kendall & Sabbadini, 1981), such as interleukin-2 (IL-2) (Farrar, Johnson & Farrar, 1981), which act as growth factors and other signals between responding cells (Andrus, Granelli-Piperno & Reich, 1984; Taniguchi *et al.*, 1986). It has been reported that administration of exogenous IL-2 limits the replication of macrophage-associated mycobacteria using *in vitro* (Bucklin & Crowle, 1986) and *in vivo* (Jeevan and Asherson, 1988) models; however, the exact mechanism by which this occurs has yet to be defined.

We have previously shown (Orme, Ratcliffe & Collins, 1984) that, at the peak of primary immunity to BCG infection, the animal acquires a population of T cells that is capable of actively utilizing or absorbing IL-2 from IL-2-containing supernatants. Since the animal at that time was expressing high levels of acquired resistance, we hypothesized that the IL-2 absorbing T cells, and the T cells expressing protective immunity, might represent the same population of cells.

Others, however, have not reached the same conclusions. For example, Turcotte and colleagues (Turcotte, 1981; Turcotte & Lemieux, 1982; Turcotte & LeGault, 1986) have reported that populations of suppressor T cells generated in response to BCG infection inhibit Con A-stimulated T cells from producing IL-2; moreover, Colizzi *et al.* (1984) have reported that non-specific factors are released from BCG-immune T lymphocytes and macrophages, which suppress the production of IL-2 from Con A-stimulated T cells.

The purpose of the present report was to further evaluate the association between the kinetics of IL-2 production and utilization (or at least absorption of IL-2 by IL-2 receptors) and the type of immunity expressed in the BCG-infected animal at the time of cell harvest. The results will show that mitogen-induced levels of IL-2 were severely depressed early during the course of the infection, when the host was in a state of active immunity, and that T cells harvested at this time rapidly utilized IL-2 from recombinant IL-2-containing supernatants. On the other hand, a recovery in measurable levels of mitogen-induced IL-2 could be detected by Day 25 of the infection, at a time when immunity was increasingly expressed by cyclophosphamide-resistant memory T cells.

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MATERIALS AND METHODS

Mice

Specific pathogen-free female B6D2 (C57BL/6 × DBA/2) F_1 hybrids were purchased from the Trudeau Animal Breeding Facility, Saranac Lake, NY. They were housed under barrier conditions, given sterile food and drinking water *ad libitum*, and used between the ages of 6 and 15 weeks.

Bacteria

M. bovis BCG Pasteur (Trudeau Mycobacterial Culture Collection strain 1011) and *M. tuberculosis* Erdman (TMCC 107) were grown in Proskauer-Beck medium containing 0·1% Tween 80 as previously described (Orme, 1987). Cultures were frozen at −70° in 1-ml aliquots until required.

Experimental infections

Within 1 hr prior to use, bacterial cultures were thawed rapidly and sonicated for 20 seconds in a Branson 1200 water bath sonicator (Branson Cleaning Equipment Co., Shelton, CT). Mice were infected i.v. via a lateral tail vein with 7×10^7 viable *M. bovis* BCG or 1×10^5 virulent *M. turberculosis* Erdman suspended in 0·2 ml phosphate-buffered saline (PBS).

Assay of resistance to tuberculosis by using passive cell transfer

Mice infected with BCG were killed at specified times, and harvested splenocytes were enriched for T cells by a panning procedure as described previously (Orme & Collins, 1983).

To enrich for T-cell subsets, the following mAb (purchased from ATCC) were used: hybridoma F7D5 (anti-Thy-1.2), hybridoma GK1.5 (anti-L3T4) and hybridoma TIB 150 (anti-Lyt-2.2). T-cell-enriched splenocytes (5×10^7/ml) were incubated with 1/5 dilutions of mAb plus 1 ml Low-Tox-M rabbit complement (Accurate Chemical and Scientific, Westbury, NY), in a one-step procedure for 60 min at 37°, and then were washed twice before further use. Assessment of the purity of T-cell subsets was performed as described previously (Orme, 1987).

The enriched T cells were infused i.v. in one-spleen equivalents into sex- and age-matched syngeneic recipients that had been exposed to 500 rads of ionizing gamma-irradiation (^{137}Ce source at a dose rate of 80 rads/min) 24 hr earlier. Mice were then challenged with 1×10^5 viable *M. tuberculosis* Erdman, and the numbers of viable tubercle bacilli were determined 10 days later. Growth of BCG was prevented on 7H11 media (Gibco, Grand Island, NY) by incorporating 1 μg/ml 2-thiophenecarboxylic acid hydrazide.

Acquired specific resistance was expressed as the $\log_{10}$ difference between the mean numbers of *M. tuberculosis* in the target organs of control mice and those which previously received BCG.

In some experiments, T-cell donors were pretreated with an i.v. inoculum of 150 mg/kg cyclophosphamide (Cytoxan, Mead-Johnson, Evansville, IL) 1 hr before cell harvest. Memory T cells, but not actively immune T cells, are resistant to this treatment (Orme, 1988a).

IL-2 assays

Spleens from normal or BCG-infected donors were removed aseptically, teased through stainless steel mesh, and washed in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) containing 1 mM glutamine, 200 U/ml penicillin, 200 μg/ml streptomycin, 25 μg/ml gentamicin, 0·5 μg/ml amphotericin B, 5×10^{-5} M 2-mercaptoethanol, and 2% heat-inactivated fetal calf serum (Sigma Chemical Co., St Louis, MO). Splenocytes were dispersed into four samples: untreated, non-adherent enriched (after panning), L3T4$^+$-enriched, and Lyt-2$^+$-enriched (after mAb treatment) splenocytes. Cells were diluted to 5×10^6/ml in the same medium, but containing 10% heat-inactivated fetal calf serum, to which 4 μg/ml Con A (Sigma Chemical Co., St Louis, MO) were added. Following a 24-hr incubation period at 37° in 5–7% CO_2-enriched humidified air, cells were centrifuged at 400 *g* for 10 min. The supernatants were passed through a sterile 0·2 μm pore diameter filter unit (Vangard International, Neptune, NJ), and assayed for IL-2 activity.

IL-2 activity was determined by using the IL-2-dependent HT-2 cell line (courtesy of Dr P. Marrack). Triplicate two-fold serial dilutions of supernatants containing IL-2 were plated in 96-well microtitre plates. HT-2 cells (3×10^4/well) were added to give a final volume of 100 μl/well, and cultured for 24 hr at 37° in 5–7% CO_2-enriched humidified air. Cells then were pulsed with 1·0 μCi of tritiated thymidine (Specific activity 6·7 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) for 6 hr and then harvested on glass fibre filters with a Brandel Standard Cell Harvester (Brandel Biomedical Research and Development Laboratories, Gettysburg, MD). Uptake of radioactive label was measured with an LKB liquid scintillation counter. For negative controls, HT-2 cells were cultured in medium devoid of IL-2. The mAb S4B6.1 (anti-IL-2) and PC61.5.3 (anti-IL-2 receptor) reduced HT-2 proliferation by mitogen-induced supernatants and in supernatants containing recombinant IL-2 to background levels, demonstrating the bioassay to be specific for IL-2. Recombinant murine IL-2 (Genzyme, Boston, MA) was used as a standard for supernatants containing IL-2.

To test for IL-2 absorption by L3T4$^+$- and Lyt-2$^+$-enriched T cells isolated from BCG-infected mice, such cells (5×10^5/well) were cultured in 100 μl media containing 5 U/ml rIL-2. Five U/ml rIL-2 were determined optimal for this assay procedure by preliminary titrations. Supernatants were withdrawn 1, 4, 8, 12,

Table 1. Passive transfer of acquired specific resistance

Day	$\log_{10}$ viable bacteria/spleen	$\log_{10}$ transferred resistance* Immune T cells†	Cy^R	$\%Cy^R$
1	3·9	0·00	0·00	—
5	5·3	0·22‡	0·08‡	—
10	6·8	1·28	0·17‡	—
15	6·4	1·85	0·70	38
20	6·0	1·77	1·05	59
25	5·8	1·96	1·73	88
30	5·7	1·69	0·82	48
40	5·5	1·81	1·53	85

* For each passive cell transfer, $n = 4$.

† T cells from BCG-infected mice not exposed to cyclophosphamide (Cy) therapy, representing both cyclophosphamide resistant (Cy^R) and sensitive T cells.

‡ Insignificant amount of resistance transferred.

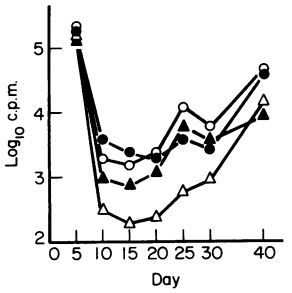

Figure 1. Mitogen-induced IL-2 activities of T cells in mice heavily infected with *M. bovis* BCG. At the time points indicated, 5×10^5 splenocytes/ml were incubated with 4 μg/ml of Con A, and IL-2 activity was determined using the HT-2 cell line. (○) Unfractionated splenocytes; (●) non-adherent splenocytes; (▲) Lyt-2$^+$-enriched T cells; (△) L3T4$^+$-enriched T cells. Results are expressed as the means of triplicate cultures.

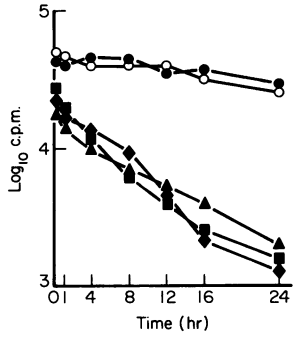

Figure 2. IL-2 absorption by BCG-immune T cells. T cells were harvested from mice that were (○) uninfected or BCG-infected, (●) Day 25 cyclophosphamide-pretreated, (■) Day 10 immune T cells, (◆) Day 20 immune T cells and (▲) Day 40 immune T cells. T cells (5×10^5 cells/ml) were placed in 100 μl of media containing 5 U/ml of rIL-2. At the time-points indicated, supernatants were extracted and assayed for IL-2 activity. Results are expressed as the means of triplicate cultures.

16, and 24 hr following the addition of the enriched T-cell subset to the rIL-2, and were assayed for IL-2 activity using HT-2 cells.

RESULTS

Development of specific resistance in BCG-infected mice

To evaluate the levels of specific resistance developed during the BCG infection, splenic T cells were transferred to sublethally irradiated syngeneic recipients, who were then challenged with an inoculum of 1×10^5 virulent *M. tuberculosis*. Table 1 shows that specific resistance had developed by 10 days following BCG infection, and remained at significant levels throughout the study.

To determine the presence of memory T cells within the infected host at these times, donor animals were pretreated with cyclophosphamide (Orme, 1988a, b). It was found (Table 1) that such cells were present by Day 15 of the infection, and became the predominant phenotype by Day 25. The percentage of cyclophosphamide-resistant (Cy^R) cells declined between Days 30 and 35 post-infection, as we have observed previously (Orme, 1988b), but returned to predominant levels by Day 40.

Levels of mitogen-induced IL-2

To determine levels of mitogen-induced IL-2 during the course of the BCG infection, fractionated splenocytes were exposed to Con A for 24 hr, after which the supernatants for each fraction were evaluated for IL-2 activity. Detectable levels of IL-2 were severely depressed between Days 10 and 20 of the infection (Fig. 1), but increased by Day 40 of the infection. At this time, all four populations of cells tested showed evidence of greater than 50% recovery towards pre-infection levels.

Ability of harvested T cells to absorb IL-2

To evaluate the ability of immune T cells to absorb exogenous IL-2, harvested T lymphocytes were placed in medium containing 5 U/ml recombinant IL-2, and samples of supernatants were withdrawn at various timepoints thereafter and tested for their ability to support the proliferation of the HT-2 cell line. As is shown in Fig. 2, it was found that enriched T cells at all times of the BCG infection contained cells capable of absorbing IL-2. In contrast, cyclophosphamide-resistant T cells from such animals did not have this absorptive capacity.

DISCUSSION

The results of this study show that in mice heavily infected with *Mycobacterium bovis* BCG Pasteur, patterns of IL-2 production and utilization are temporally related to the type of immunity that is predominantly expressed in response to the infection. Thus, when active immunity was the predominant form of immunity being expressed, mitogen-induced levels of IL-2 measured *in vitro* were low or negligible, and we show here that this is associated with the ability of both L3T4$^+$ and Lyt-2$^+$ T cells from these animals to absorb IL-2 from IL-2-containing media. This correlation, moreover, is further consistent with the observation that actively immune T cells are both irradiation- and cyclophosphamide-sensitive, indicating they are IL-2-dependent, rapidly expanding, proliferating populations of cells (Orme, 1987).

As the course of the BCG infection progressed, however, more and more immune T cells expressed a cyclophosphamide-resistant phenotype, which we have previously shown is a characteristic of memory immune T cells (Orme, 1988a, b). Thus, by Day 40 of the infection, memory T cells accounted for 85% of transferred resistance using the passive cell transfer assay; moreover, none of these cells were able to absorb IL-2 *in vitro*. A residual population of T cells capable of absorbing IL-2 in the *in vitro* assay clearly still remained at this time, however, which presumably explained the incomplete recovery of IL-2 levels following mitogen stimulation at this time-point (Fig. 2).

Collectively, these data indicate that levels of IL-2 that can be measured in the supernatants of mitogen-stimulated spleen cell preparations from mycobacteria-infected mice will be subject to fluctuations as a result of the binding of IL-2 by T-cell populations that are present at various times during the infection. Thus, mitogen-induced levels of IL-2 will be expected to be low when many T cells, activated by the BCG infection, are expressing IL-2 receptors (which they do with very high frequency; I. M. Orme, M. J. H. Ratcliffe, unpublished data), which will serve to rapidly absorb the produced IL-2. On the other hand, in situations in which more memory immunity than active immunity predominates, less absorption of mitogen-

induced IL-2 would be expected to occur. This is borne out in the present report, in which predominant numbers of memory immune T cells were associated with a gradual recovery in the levels of IL-2 induced by mitogen stimulation.

In summary, this report provides evidence that in mice heavily infected with BCG, a shift in the form of immunity being expressed, from active immunity to a state of immunological memory, correlates with a recovery in the levels of IL-2 that could be induced by mitogen stimulation of spleen cells from the BCG-infected animals. These results thus support the hypothesis that in high-dose BCG infection, the levels of mitogen-induced IL-2 that can be measured reflect the characteristics of the T-cell population mediating the response to the infection at that time, rather than indicating some form of defect in or suppression of IL-2 production.

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