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Suppressor cells induced by BCG release non-specific factors *in vitro* which inhibit DNA synthesis and interleukin-2 production

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Summary. Mice injected intravenously with a high dose (5×10^7) of BCG fail to develop delayed hypersensitivity to BCG and are described as anergic or unresponsive. Spleen cells from these mice release factors on culture which suppress DNA synthesis induced by concanavalin A *in vitro*. Cell separation experiments showed that both macrophages and T cells produce inhibitory factors. However, the macrophage factor has a molecular weight 10,000–30,000, while the T cell factor has a molecular weight of 50,000–70,000. Further evidence that these two factors are different is provided by the kinetics of their action. The T cell factor only acts when given within 12 hr of stimulation with concanavalin A, while the macrophage factor acts even when given at 48 hr.

In the case of the T cell factor, the inhibition of DNA synthesis may be attributed to its ability to block the interleukin-2 production induced by Con A. As similar T cell and macrophage factors are produced in mice responding to simple chemically reactive haptenes (contact sensitizers), it is possible that a similar suppressor circuit is involved in the control of the response to contact sensitizers and in the production of unresponsiveness (anergy) in mice given large doses of BCG.

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INTRODUCTION

Miliary tuberculosis in humans is associated with non-specific loss of delayed hypersensitivity skin reactions (Lamoureux & Poisson, 1974). Similarly, intravenous injection of high doses of BCG causes a non-specific loss of cell-mediated immunity in mice (Bullock, 1975). This anergy in mice affects delayed hypersensitivity and the graft-versus-host response *in vivo*, and alloantigen- and mitogen-induced blast transformation *in vitro* (Gefford & Orbach-Arbouys, 1976; Florentin & Orbach-Arbouys, 1977; Collins, Morrison & Montalbine, 1978; Collins & Watson, 1979). Suppressor cells are responsible, at least in part, for this state of anergy. For instance, Collins & Watson (1979) showed that mice infected with large doses of BCG developed T suppressor cells in the spleen which blocked mitogen- and antigen-induced lymphocyte proliferation *in vitro* and that these cells appeared at the time when the mice lost their delayed hypersensitivity skin reactions. More recently, Turcotte & Lemieux (1982) found that T cells and macrophages from anergic BCG-infected mice produced soluble factors which depressed DNA synthesis.

There is considerable knowledge of the control of the response to simple chemically reactive compounds such as picryl chloride. It seemed of interest to compare the mechanisms which affect contact sensitivity to simple haptenes with the mechanism of unres-

ponsiveness in mice infected with BCG. In the picryl system, the spleen from mice injected with picryl sulphonic acid contains antigen-specific T suppressor cells. These cells produced an antigen-specific suppressor factor which does not affect the immune response directly, but indirectly by arming other cells called non-specific acceptor cells. These cells, which may be macrophages (Ptak *et al.*, 1981) or T cells (Asherson & Zembala, 1982; Sy *et al.*, 1979), lack intrinsic relevant immunological specificity but acquire specificity when armed with the antigen-specific T suppressor factor. Subsequent exposure to antigen together with major histocompatibility complex (MHC) products causes the release of non-specific inhibitor(s), which block the passive transfer of contact sensitivity, depress lymphocyte proliferation and reduce interleukin-2 (IL-2) production (Ptak *et al.*, 1981; Zembala *et al.*, 1982; Asherson *et al.*, 1982; Malkovsky *et al.*, 1982, 1983).

This paper describes similar non-specific inhibitors of DNA synthesis and IL-2 production in mice rendered anergic with high doses of BCG. At least two factors are produced, one by T cells and the other by macrophages.

MATERIALS AND METHODS

Infection of mice

CBA mice, bred at the CRC, were injected intravenously (i.v.) with 5×10^7 viable BCG cells (*Mycobacterium bovis*, strain BCG, kindly provided by Glaxo, Middlesex, U.K.) or intradermally (i.d.) into the right footpad with 5×10^6 BCG cells.

Delayed hypersensitivity footpad reaction to PPD

Fourteen days after i.v. or i.d. injection of BCG, the mice (5 per group) were challenged by injecting PPD (purified protein derivative; Central Veterinary Laboratory, Weybridge, Surrey, U.K.), 20 μg in 0·05 ml of saline into the left hind footpad. The increase in the footpad thickness between 0 and 24 hr was measured with a dial micrometer and expressed in units of 10^{-3} cm. The negative control group consisted of uninfected mice challenged with PPD.

Cell separation

Spleen cells were taken from mice 14 days after intravenous injection of BCG and suspended in RPMI-1640 (Flow Laboratories) supplemented with penicillin, streptomycin and glutamine and 10% inactivated foetal calf serum. The macrophage-enriched population was obtained by adding spleen cells (5×10^7 in 10 ml) to bacteriological Petri dishes (9 cm) previously coated with 20% inactivated newborn calf serum for 1 hr, and incubated at 37° in 5% CO_2/air for 1 hr. The adherent cells were rubbed off from the plate and provided the crude macrophage enriched population. To remove any T cell contamination they were then treated with monoclonal anti-Thy-1.2 antibody (Olac) and rabbit complement (1:5).

B cells were recovered from the macrophage depleted population by panning on petri-dishes coated with $F(ab')_2$ rabbit anti-mouse Ig (Zembala *et al.*, 1982). The recovery was 45% of the applied cells. T cells were then positively selected from the population depleted of macrophages and B cells by treating 10^8 cells in 1 ml with an equal volume of 1/100 monoclonal anti-Thy-1.2 at 4° for 1 hr, diluting, washing twice and then panning on petri-dishes coated with rabbit $F(ab')_2$ rabbit anti-mouse Ig for 1 hr at room temperature. The recovery was 80% and these were regarded as T cells.

Preparation of supernatants

The crude spleen cells or purified cell populations were then suspended in medium at 10^7/ml and incubated for 24 or 48 hr. The supernatants were collected and filtered through a 0·22 μm Millex-Gs Millipore filter.

Assay of inhibition of DNA synthesis

Spleen cells from normal CBA mice (10^6 cells/ml) were cultured in 0·2 ml medium with 3 μg/ml Concanavalin A (Con A; Pharmacia) in triplicate in 96-well Titertek tissue culture plates. In general the final dilution of the supernatant in the assay was 1/2 (50%). At 67 hr 1 μCi (methyl-[^{3}H])-thymidine ([^{3}H]TdR; sp.act. 5 Ci/mM; TRA 120, Radiochemical Centre, Amersham, U.K.) was added and the plates harvested 5 hr later with a cell harvester. [^{3}H]-thymidine incorporation was determined by liquid scintillation counting.

Assay of inhibition of IL-2 production

A lymphoid cell suspension was prepared by pressing CBA spleens through a fine mesh screen into RPMI-1640 medium. Erythrocytes were lysed using Boyle's solution. The lymphoid cells were mixed with a 50% concentration of the supernatant or with 50% of the inhibitory control supernatant (10^7 cells/ml); Con A (5 μg/ml) was added and the suspension was distributed in 4 ml aliquots into 12-well flat-bottom Linbro tissue culture plates (Flow). Similarly, 4 ml aliquots of the same cells (10^7 cells/ml) were resus-

pended in RPMI-1640 medium with (positive assay control) or without (negative assay control) Con A (5 μg/ml), and distributed into the plates. After 4 hr incubation, the supernatant was carefully removed and discarded. Fresh serum-free RPMI-1640 medium (without Con A) was added and the supernatant was collected after a further incubation for 20 hr and tested for IL-2 activity. The presence of IL-2 was measured using 4 day Con A blast cells essentially following the method of Granelli-Piperno, Vassali & Reich (1981). CBA Con A blast cells (10^6 cells/ml) were cultured in 0·2 ml supplemented RPMI-1640 medium in the presence of various concentrations of the supernatants to be tested for IL-2 activity. Cultures were set up in quadruplicate in 96-well Titertek tissue culture plates. Five hours before collection, each well was pulsed with 1 μCi [^{3}H]TdR. The cultures were collected after 24 hr and [^{3}H]TdR incorporation was determined.

Sephadex gel filtration

A Sephadex G-100 column (3 × 80 cm) was run at 10 ml/hr. The column was calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen A, and cytochrome C. Inhibitory supernatants (20 ml) obtained after 24 hr culture of whole spleen cells, or macrophage-enriched or T cell-enriched cell population from i.v. BCG-infected mice, were concentrated on YM10 membranes to 4 ml. Fractions were pooled, concentrated by an Amicon PM50 or YM10 membrane to 4 ml, and finally dialysed against medium.

Statistical analysis

Data are expressed as mean ± standard deviation. The double-tailed Student's *t*-test was used.

RESULTS

Inhibition of Con A-induced DNA synthesis by culture supernatants of spleen cells from anergic BCG-infected mice

Mice infected with high doses of BCG intravenously showed minimal reactions to footpad challenge with PPD 14 days later (Table 1). In contrast, mice infected with low doses of BCG intradermally exhibited a strong delayed hypersensitivity reaction to PPD.

The spleen cells from these responsive and unresponsive groups were then cultured *in vitro* for 24 hr and the appearance of inhibitory factor(s) in the supernatant was studied. Table 2 shows that the supernatant of cells from unresponsive mice blocked con A-induced DNA synthesis while supernatant from responsive mice failed to inhibit. Control supernatant from uninfected mice also failed to inhibit.

Gel filtration showed that the inhibitory activity occurred in two distinct areas between 50,000–70,000 and 10,000–30,000 (Fig. 1).

Table 1. Delayed type hypersensitivity skin reactions to PPD in BCG-infected mice

Route of injection	BCG doses	DTH to PPD
i.v.	5×10^7	12·8 ± 3·5
i.d.	1×10^7	42·3 ± 13·4
Uninfected	(negative control)	5·0 ± 2·7

* Mice were injected with viable BCG cells in the right hind footpad or intravenously.

† Mice were challenged 14 days after infection by injecting 20 μg PPD into the left footpad. The increase in the footpad was measured 24 hr later and expressed in units of 10^{-3} cm ± SD.

Table 2. Effects of supernatants from BCG-infected mice on Con A-induced DNA synthesis

Supernatants from	Con A-induced [^{3}H]TdR incorporation (c.p.m. ± SD)
—	90,724 ± 10,412
Uninfected	85,392 ± 5427
i.v. BCG	11,413 ± 3411
i.d. BCG	83,944 ± 11,432

* Supernatants (0·1 ml) prepared by culturing spleen cells from normal or BCG-infected mice (10^7 cells/ml for 24 hr) were added to normal spleen cells (2×10^5 in 0·1 ml) which were then stimulated with Con A. The cells were harvested on Day 3.

Nature of cells producing non-specific inhibitors of DNA synthesis

The molecular weight studies suggested that at least two distinct molecules were produced. It is known that both macrophages and T acceptor cells make non-specific inhibitor in a contact sensitivity system (Ptak *et al.*, 1978; Zembala *et al.*, 1982). The question therefore arose whether these two distinct cells produced the two factors in the present system.

The spleen cells of unresponsive mice were separated into macrophages (plastic-adherent), B cells (Ig-positive) and T cells by a sequential procedure. The

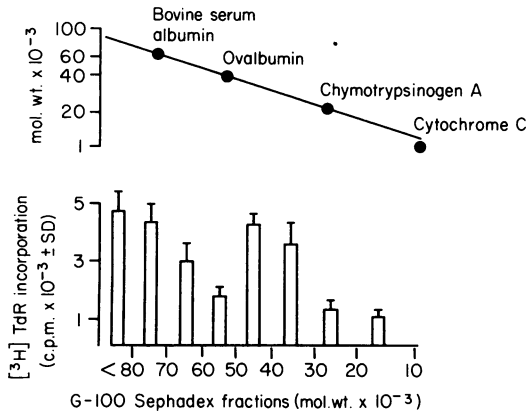

Figure 1. Molecular weight of non-specific inhibitors produced by spleen cells from mice infected with high doses of BCG. The non-specific inhibitors were separated on Sephadex G-100. The resulting fractions were pooled in molecular weight bands, concentrated to 4 ml, and assayed for their ability to depress Con A-induced DNA synthesis.

T cells were then further purified by panning using a monoclonal anti-Thy-1.2 serum, while the macrophages were further purified by treating with anti-Thy-1.2 serum and complement. The culture supernatants from these different cell populations were then tested for their ability to inhibit DNA synthesis. Figure 2 shows that both T cells and macrophages produced non-specific inhibitors while the B cells were inactive.

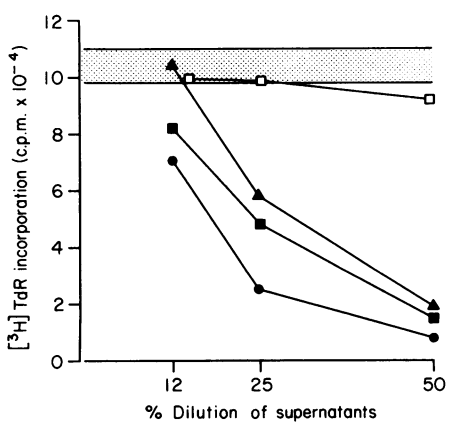

Figure 2. Nature of cells producing nonspecific inhibitors. Unseparated spleen cells (■), macrophages (●), B cells (□), or T cells (▲), from i.v. BCG-infected mice were cultured as described in 'Materials and Methods'. The culture supernatants were tested for their capacity to influence Con A-induced DNA synthesis. The *dotted area* indicates the range of Con A-induced DNA synthesis in cells incubated in culture medium only.

The molecular weight of the non-specific inhibitor produced by T cells and macrophages was then determined. Figure 3 confirms the pattern seen in Fig. 1 and shows that the T cell non-specific inhibitor had a molecular weight of 50,000–70,000, while the macrophage inhibitor had a molecular weight of 10,000–30,000.

The time course of the action of the inhibitory supernatants produced by macrophages and T cells provided further evidence that the two factors are different. Figure 4 shows that the non-specific inhibitor produced by macrophages was active when added at 12, 24 or 48 hr to Con A-stimulated cultures. In contrast, the non-specific inhibitor produced by T cells was only active when added within the first 12 hr of con A stimulation.

Inhibition of IL-2 production

The non-specific inhibitor of DNA synthesis produced by T acceptor cells armed with antigen-specific T suppressor factor blocks the production of IL-2 (Malkovsky *et al.*, 1982). In the present system the finding that the non-specific inhibitor produced by T cells only acts when added within 12 hr of the Con A stimulation suggested that the inhibition of DNA synthesis might be due at least in part to a failure of IL-2 production. To test this hypothesis the effect of various supernatants on IL-2 production induced by Con A was measured. In practice normal spleen cells were pulsed with Con A and inhibitor, and afterwards cultured in fresh medium and the production of IL-2 at 24 hr measured. Figure 5 shows that the supernatants of spleen or T cells depressed the production of IL-2, while the supernatant of B cells had no effect.

DISCUSSION

This paper demonstrates that T cells and macrophages from mice infected intravenously with high doses of BCG produce soluble factors *in vitro* which inhibit Con A-induced DNA synthesis. These cells, which release non-specific inhibitors, occur in the spleen of BCG-infected mice at the time when the animals are anergic and lack skin reactions to PPD.

It is known that mice infected intravenously with large numbers of BCG quickly develop T cells and macrophages in the spleen which are capable of

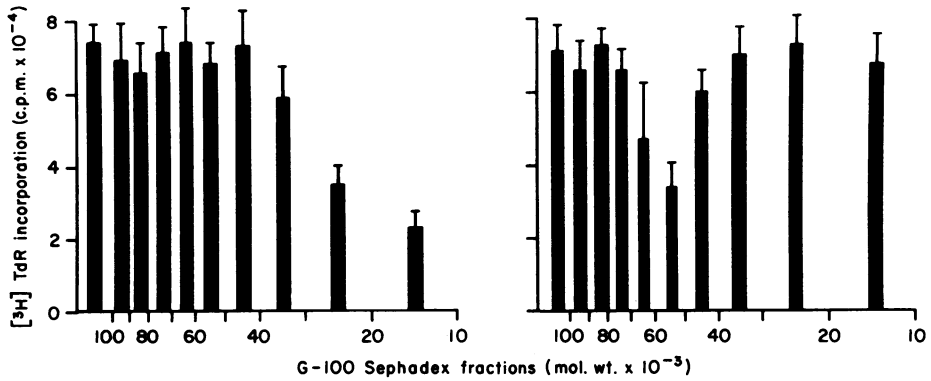

Figure 3. Molecular weight of non-specific inhibitors produced by macrophages and T cells. Macrophages or T cells were isolated from spleen cells. Their products were separated on Sephadex G-100 and the pooled fractions were tested in the DNA synthesis assay. The *left part* of the figure illustrates the molecular weight of the macrophage non-specific inhibitor, the *right part* indicates the molecular weight of the T cell non-specific inhibitor.

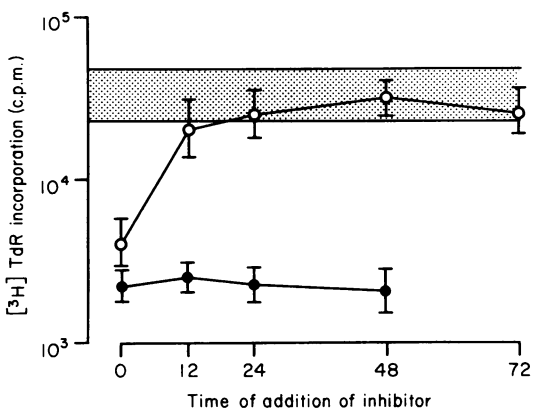

Figure 4. CBA spleen cells were seeded into 96-well Titertek tissue culture plates (2×10^5 cells/well) and were cultured in 100 μl RPMI-1640 medium supplemented for the DNA synthesis assay as described in 'Materials and Methods'. One hundred microlitres of a non-specific inhibitor-containing supernatant produced by either macrophages (●) or T lymphocytes (○) were added to cultures immediately or subsequently as indicated in the abscissa. The dotted area represents the range of Con A-induced DNA synthesis in cells incubated in medium only.

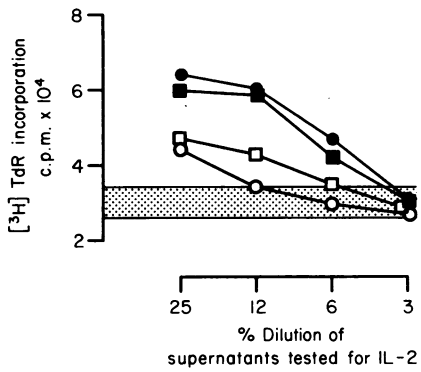

Figure 5. IL-2 activity in supernatants of Con A-activated cells exposed to control medium (●) or to non-specific inhibitor released by unseparated spleen cells (○), T cells (□) or B cells (■). The *dotted area* illustrates DNA synthesis in indicator 4-day Con A blast cells incubated in medium without adding any supernatants.

suppressing both non-specific, mitogen- and specific antigen-induced proliferative response *in vitro* (Collins & Watson, 1979; Turcotte, 1981). More recently, Turcotte & Lemieux (1982) reported that both these cell populations produce factors *in vitro* which non-specifically suppress the DNA responses to mitogens. The present experiments confirmed their findings and clearly demonstrate that at least two different factors displaying an inhibitory activity are produced by the spleen cells of mice unresponsive to BCG. These two factors have different molecular weights. The T cell product has a molecular weight of 50,000–70,000, while the macrophage factor has a molecular weight of 10,000–30,000. Moreover, the kinetics of their action is different. The inhibitory factor produced by T cells only acts when given within 12 hr of stimulation with Con A, while the inhibitory factor produced by macrophages acts even when given at 48 hr.

More detailed analysis shows that the inhibitor produced by T cells blocks the production of IL-2 as measured by the ability of IL-2 to support the

proliferation of 4-day blast cells. This inhibition of IL-2 production *in vitro* probably also occurs *in vivo* as Hoffenbach, Lagrange & Bach (1983) reported that mice infected with *Mycobacterium lepraemurium* have reduced IL-2 production. This may explain their failure to respond to mitogens and allogeneic cells *in vitro*.

The present system of unresponsiveness produced by the injection of large doses of BCG intravenously has interesting analogies to the T suppressor circuit which influences the response to contact sensitizers. In that system, antigen-specific T suppressor factor binds to macrophages and T acceptor cells. These cells then release non-specific inhibitor of the passive transfer of contact sensitivity when exposed to antigen under appropriate conditions. There is a similarity between these inhibitors and those produced by mice unresponsive to BCG. In both systems the molecular weight of the factor produced by T cells is 30,000–70,000 (Zembala *et al.*, 1982). Moreover, in both systems the factor(s) produced by T cells blocks DNA synthesis induced by Con A and this inhibition only occurs when the inhibitor is added within the first 12 hr of culture. In both cases the nonspecific inhibitor blocks IL-2 production. These findings suggest that identical nonspecific inhibitors may occur in the T suppressor circuit which regulates contact sensitivity and in mice rendered unresponsive to BCG.

It is known that the T suppressor circuit which limits the effector stage of the contact sensitivity reactions is also responsible for the phenomenon of desensitization *in vitro* (Thomas *et al.*, 1981; Asherson *et al.*, 1982), i.e. the inhibition of the transfer of contact sensitivity to picryl chloride by incubating the passive transfer population with haptene (picrylated spleen cells). In fact the three elements of this suppressor circuit—the T suppressor cell (efferent), antigen specific T suppressor factor and the T acceptor cell—occur in the population which transfers contact sensitivity and are responsible for desensitization *in vitro*. The present finding that T cells from mice infected with BCG produce non-specific inhibitors *in vitro* suggests that these mice may contain all the elements needed for desensitization, i.e. the presence of the appropriate suppressor cells and the presence of antigen in large amounts. A simple view is that mycobacterial antigen leads to the release of specific T suppressor factor which then arms the acceptor cell (whether a T acceptor cell or a macrophage), and this in turn releases non-specific inhibitors when exposed to the antigen. These nonspecific inhibitors may be responsible for the loss of delayed hypersensitivity to PPD and other antigens seen *in vivo* and the loss of mitogenic responses *in vitro*. Some support for this interpretation of anergy is provided by the finding that specific T suppressor cells occur in mice injected with high doses of BCG (Nakamura & Tokunaga, 1980).

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