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BCG-induced suppressor T cells optimal conditions for *in vitro* induction and mode of action

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SUMMARY

In vitro activation with BCG of T cells from healthy individuals vaccinated with BCG lead to the induction of suppressor cells that suppressed the proliferation of fresh T cells in response to specific antigen. Kinetics of their induction revealed that they became radioresistant by day 8 and persisted up to 18 days of the culture period. Optimal antigen and monocyte concentrations as assessed by proliferation during the induction phase also resulted in maximum suppression. The strongest suppressor activity was observed when suppressor cells were added at an early time of fresh cell activation. Il-1 production from adherent cells in response to BCG was not affected, but, IL-2 production by T-cells was considerably reduced in the presence of suppressor cells. IL-1 containing supernatants and affinity purified IL-1 exogenously added to the culture system did not affect suppression. Whereas, recombinant IL-2 partially abrogated suppression in a dose-dependent manner. Further experiments suggested that suppressor cells might have inhibited BCG induced IL-2 receptor expression on fresh T cells.

Keywords BCG suppressor cells interleukins

INTRODUCTION

Immunization against tuberculosis and leprosy has been tried using BCG vaccines. In certain areas it was protective (Brown, Stone & Sutherland, 1968; Medical Research Council, 1972; Rosenthal *et al.*, 1961) while in others it failed to give protection (Bechelli *et al.*, 1973; Comstock & Webster, 1969; Tuberculosis Prevention Trial, Madras, 1980). Many explanations have been given to explain the failure of protection (BCG vaccination after the Madras study, 1981; Brandely, Hurtrel & Lagrange, 1983; Stanford, Sheild & Rook, 1981). However, a number of studies directed at understanding the mechanism of immunodeficiency in pathological situations including mycobacterial infections have suggested the involvement of suppressor T cells (Mehra *et al.*, 1982; Piessens *et al.*, 1982; Ohta, Minai & Sasazuki, 1983). We have successfully induced antigen specific MHC restricted and $CD4^+$ suppressor T cells from PBMC of BCG-immunized healthy subjects after *in vitro* exposure to the specific antigen (Mustafa & Godal, 1983). The results reported in this article show that suppressors were induced under optimal conditions of antigen-induced T cell proliferation, become radioresistant, and could manifest their activity even up to about 3 weeks in culture.

Interleukin 1 (IL-1) and interleukin 2 (IL-2) are the known cytokines with proven roles in T cell activation and proliferation. IL-1 is a monocyte/macrophage product and is required by the T cells to produce IL-2 (Larsson, Iscove & Coutinho, 1980; Smith *et al.*, 1980). The concentration of IL-2 and the expression of IL-2 receptors on T cells will determine the extent of their proliferation and

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growth (Cantrell & Smith, 1984). In our system, BCG-induced suppressor cells did not affect IL-1 production by monocytes, but might have a major effect at the level of IL-2 production and IL-2 receptor expression by T cells.

MATERIALS AND METHODS

Reagents. Ficoll-hypaque (lymphoprep) was purchased from Nyegaard & Co., Oslo, Norway. Tissue culture medium RPMI was obtained from GIBCO Laboratories, Porisley, Scotland, UK. Pooled normal human AB serum was supplied locally by the blood bank. The complete medium (CM) contained RPMI-1640 with 15% heat-inactivated human AB serum and 1% Penicillin-Streptomycin. Live BCG from liquid cultures was obtained from Statens Serum Institute, Copenhagen, Denmark and was used at a final concentration of 20 μg/ml (wet weight). Affinity purified human IL-1 was purchased from Genzyme, Maidstone, England. Recombinant IL-2 (Rec-IL-2) was a kind gift from Cetus Corporation, California. Anti-Tac monoclonal antibody recognizing IL-2 receptors on activated T-cells (Leonard *et al.*, 1982) was a kind gift of Dr Thomas A. Waldmann, Chief Metabolism Branch, NCI, NIH, Bethesda, Maryland, USA. FITC conjugated sheep anti-mouse IgG prepared by the method of Kvaløy *et al.* (1984) was supplied by Dr T. E. Michaelsen, National Institute for Public Health, Oslo, Norway. The Pan T cell monoclonal antibody T3 was purchased from Orthopharmaceuticals, Raritan, New Jersey, USA and a human monocyte specific monoclonal antibody ID5 (Kaplan & Gaudernack, 1982) was a gift from Dr G. Gaudernack, Tissue Typing Laboratory. Rikshospitalet, Oslo, Norway.

Isolation of peripheral blood mononuclear cells (PBMC), adherent cells & T cells. PBMC were separated on lymphoprep gradient from the heparinized blood of BCG vaccinated healthy subjects showing strong LTT response to BCG. The T cells and adherent cells from PBMC were isolated as described by Mustafa & Godal (1983). The T cell enriched fraction was $>90\%$ T 3^+ and adherent cells were $\geq 85\%$ $ID5^+$. The cells for several repeat experiments were obtained from different donors.

Induction of suppressor cells (primary culture). Suppressor cells were induced *in vitro* by a method described earlier (Mustafa & Godal, 1983). Briefly, 10 ml samples of $1\text{–}2 \times 10^6$ cells/ml (PBMC or T cell enriched fraction + 10% adherent cells) in CM were cultured with BCG in 25 cm^2 tissue culture flasks (Costar) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Control cultures did not have BCG. At the end of primary culture, cells were washed, irradiated with 2,400 rad and assayed for suppressor activity. In experiments where primary cultures lasted for more than 8 days, viable cells were recovered on lymphoprep gradient.

Assay of suppressor cell activity (secondary culture). 1×10^5 fresh cells (PBMC or T cell enriched fraction with 10% adherent cells) were seeded in to each well of the 96-well round-bottom microtitre plates (Costar). 50 μl of BCG suspension (80 μg/ml) and 1×10^5 irradiated suppressor or control cells were added to the wells in triplicate. The culture volume was brought up to 200 μl by addition of complete medium. The plates were incubated for 6 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, and harvested after a 4-h pulse of 0.045 MBq ^{3}H-thymidine (specific activity = 185×10^3 MBq/m mole). The incorporated radioactivity was determined by liquid scintillation spectroscopy. The median values of counts per minute (ct/min) from triplicates were used to calculate percentage suppression which is defined as:

$$\text{Suppression (\%)} = \left[1 - \frac{\text{ct/min of cultures with fresh cells + irradiated cells precultured with BCG + BCG}}{\text{ct/min of cultures with fresh cells + irradiated cells precultured without BCG + BCG}}\right] \times 100$$

IL 1 production and assay. Adherent cells from 5×10^6 PBMC in each well of 12-well Costar plates were incubated with or without BCG in 2 ml CM at 37°C in an atmosphere of 5% CO_2 and 95% air. After 24 h, the cell free supernatants were collected, filter sterilized and stored frozen at

−20°C until assayed for IL-1 activity. The effect of suppressor cells on IL-1 production by adherent cells was determined by the addition of 1×10^6/ml irradiated suppressor or control cells.

IL-1 activity in the supernatants was assayed by the enhancement of C57BL/6 mouse thymocyte proliferation at a sub-optimal dose of Con A (1 μg/ml) by the method of Palacios *et al.* (1982). On day 3, the cultures were harvested after a 4-h pulse of 0·045 MBq ^{3}H-thymidine.

IL-2 production and assay. 2 ml samples of 2×10^6 fresh cells/ml in CM were placed in 10 ml plastic tubes. 1×10^6/ml irradiated suppressor or control cells were added to determine their effect on IL-2 production. BCG was added at a final concentration of 20 μg/ml. The tubes were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. 24 h later, cell-free supernatants were harvested, filtered through millipore membranes and stored at −20°C until used.

A PHA initiated IL-2 responding T cell line was used to assay IL-2 activity in the supernatants. 10^4 cells were placed into each well of 96-well, flat-bottom microtitre plates. To this were added 100 μl of supernatants; the plates were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cultures were harvested after a 4 h pulse of 0·045 MBq ^{3}H-thymidine (specific activity = 185×10^3 MBq/m mole). The incorporated radioactivity was determined by liquid scintillation spectroscopy.

Responsiveness of cells from secondary cultures to IL2 and flow cytometry. 1×10^6/ml fresh cells in CM were stimulated with BCG in 10 ml plastic tubes. 1×10^6/ml irradiated suppressor or control cells were added. The total culture volume was 5 ml. The tubes were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. On day 6, viable cells were recovered by floatation on Ficoll-hypaque. 1×10^5 viable cells were added to each well of a flat-bottom microtitre plate, and responsiveness to IL-2 was determined as described by Reis and Shevach (1982). For flow cytometry, 1×10^6 viable cells were incubated for 30 min at 4°C with anti-Tac antibody (1:5000 dilution of ascites) washed three times and reincubated with 1/40 dilution of fluorescin conjugated sheep anti-mouse IgG for 30 min at 4°C. The cells were washed and the percentage positive cells were determined by a laboratory-built flow cytometer as described by Godal *et al.* (1981).

RESULTS

BCG-induced in vitro *activation of suppressor T cells*

Suppressor T cells capable of suppressing BCG-induced proliferation of autologous fresh cells were induced *in vitro* by preculture of a T cell-enriched population with BCG (Mustafa & Godal, 1983). Most of the suppressor activity was radiosensitive when assayed after 3– to 5–days of presensitization. The cells from 8, 11 and 18 days of primary culture were suppressive in both irradiated and non-irradiated forms (Fig. 1). BCG activation of pre-irradiated T cells during primary culture did not lead to suppressor-cell induction (data not shown).

The concentrations of BCG optimal in a proliferative assay (2–20 μg/ml) were also found to activate suppressor cells (Fig. 2a). T cell preparations supplemented with 1–25% monocytes during primary culture resulted in optimal suppression (Fig. 2b). Suppressor cells were highly effective when added within 4 h of fresh cell activation (mean suppression = 75%) whereas, their effect was greatly reduced (mean suppression = 30%) when added 16–24 h later (Fig. 2c). The T cell-enriched fraction precultured with BCG for 11 days was divided into two parts. One was incubated with and the other without BCG for another 1– to 2–weeks at 37°C. Afterwards, when assayed for suppressor activity, no difference could be found in their suppressive effect (data not shown). Thus it could be said that suppressor cells in this system, once induced, did not require continuous presence of antigen for the maintenance of their activity.

Suppressor cell effect on cytokine production

Experiments were carried out to examine the effect of suppressor cells on BCG-induced IL-1 production by monocyte enriched adherent cells and IL-2 production by T cells. Addition of irradiated suppressor or control cells did not affect the level of IL-1 produced (Table 1). However, IL-2 dependent indicator cells incorporated significantly reduced radioactivity when cultured in the

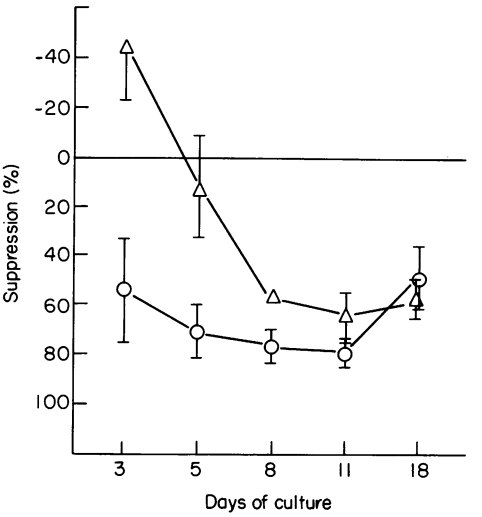

Fig. 1. Irradiation sensitivity of suppressor cells. 2×10^6/ml T cells with 10% adherent cells were cultured with BCG (20 μg/ml) or without BCG for indicated days. The precultured cells were added to BCG stimulated fresh cells in a ratio of 1:1 either after irradiation with 2,400 rad (△) or non-irradiated (○). The cultures were pulsed with ^{3}H-thymidine on day 6 and harvested 4 h later. Radioactivity incorporated was determined by liquid scintillation spectroscopy.

$$\text{Suppression (\%)} = \left[1 - \frac{\text{ct/min in cultures with fresh cells + BCG + cells precultured with BCG}}{\text{ct/min in cultures with fresh cells + BCG + cells precultured without BCG}}\right] \times 100$$

Results are the mean ± s.e.m. of four experiments from different individuals.

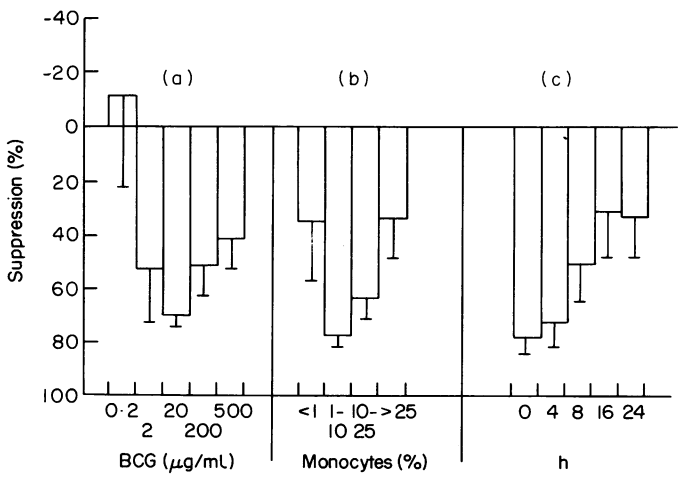

Fig. 2. Optimal conditions for suppressor cell induction and the kinetics of their effect. (a) and (b) show the effect of BCG and monocyte concentrations, respectively, on suppressor-cell induction. (c) represents the time in hours when suppressor cells were added to the fresh cells after their stimulation with BCG. Results are the mean ± s.e.m. of 4–6 independent experiments.

Table 1. Effect of suppressor cells on BCG-induced IL-1 production by adherent cells

	IL-1 activity of 24 h supernatants obtained from			
Experiment no.	Adherent cells alone	Adherent cells + BCG (mean ct/min ± s.e.m.)	Adherent cells + BCG + ir. control cells	Adherent cells + BCG + ir. suppressor cells
1	11930 ± 655	32362 ± 3940	42432 ± 1057	35039 ± 2139
2	8266 ± 1002	33585 ± 2030	41142 ± 1555	31946 ± 1830
3	8091 ± 123	42889 ± 3527	51486 ± 2796	42363 ± 4936
4	1316 ± 223	9273 ± 244	10791 ± 981	6364 ± 1315
Mean ± s.e.m.	7400 ± 2212	29527 ± 7148	36462 ± 8861	28928 ± 7832

1×10^6 thymocytes were added into each well of a 96-well flat-bottom microtitre plate. 100 μl of the supernatants were added in triplicates, and IL-1 activity was determined as described in materials and methods. Ct/min in the cultures with, (a) thymocytes alone was < 350, (b) thymocytes + Con A, was 700–800, and (c) thymocytes + IL-1 supernatants was ≤ 3000.

presence of the 24-h supernatants from BCG-stimulated fresh cells + suppressor cells as compared to the supernatants from fresh cells alone or fresh cells + control cells (Table 2).

Effect of exogenous IL-1 and IL-2 on suppression

Addition of BCG-induced IL-1 containing supernatant or affinity purified human IL-1 did not have any effect on suppression. 73% suppression was observed in the absence, and 73% and 75% in the presence of 20 u of BCG-induced IL-1 and affinity-purified IL-1, respectively. To investigate the effect of exogenous IL-2 on suppression, Rec-IL-2 was used at three different concentrations, i.e. 2

Table 2. Effect of suppressor cells on BCG-induced IL-2 production by fresh cells

	IL-2 activity of 24 h supernatants obtained from		
	A	B	C
Experiment no.	Fresh cells + BCG (mean ct/min ± s.e.m.)	Fresh cells + ir. control cells + BCG	Fresh cells + ir. suppressor cells + BCG
1	8583 ± 128	15213 ± 1296	2989 ± 188
2	8953 ± 1058	16121 ± 1244	2702 ± 144
3	8502 ± 348	7969 ± 357	3438 ± 226
4	9696 ± 194	14509 ± 374	1937 ± 169
5	10423 ± 372	13618 ± 778	5168 ± 189
Mean ± s.e.m.	9231 ± 365	13486 ± 1439	3246 ± 538

Results from the triplicates are tabulated in terms of mean ct/min ± s.e.m. The cell line used to assay IL-2 activity in the supernatants incorporated < 500 ct/min when incubated alone.

Statistical analysis by Student's *t*-test: A versus B, $P > 0.05$; A versus C, $P < 0.001$; B versus C, $P < 0.001$.

Table 3. Effect of Rec-IL-2 on the suppressive activity of BCG-induced suppressor cells

Rec-IL-2 added (u/ml*)	PBMC	PBMC+BCG	PBMC+ir. control cells (ct/min × 10^{-3})	PBMC+ir. control cells+BCG	PBMC+ir. suppressor cells+BCG	Suppression (%)
—	1±1	16±3	1±0	23±7	8±3	65
2	nd	nd	3±0	26±7	11±3	58
10	nd	nd	17±1	30±3	19±3	37
50	nd	nd	45±6	58±3	41±3	29

* 1 u of IL-2 was the concentration that resulted in 50% of the maximum response of IL-2-dependent T cell line in 24 h assay.

The results are mean±s.e.m. of three individual experiments.

nd = Not done

u/ml, 10 u/ml and 50 u/ml (Table 3). PBMC proliferation increased with increasing concentrations of Rec-IL-2, and the net effect was partial abrogation of suppression in a dose-dependent manner. At the highest concentration of Rec-IL-2, minimal suppression was observed.

Effect of suppressor cells on IL-2 receptor expression on fresh T cells

The effect of irradiated suppressor cells on IL-2 receptor expression by fresh T cells in response to BCG was studied by determining the IL-2 responsiveness and anti-Tac positivity of the cells on day 6 of secondary cultures. The IL-2 response of cells cultured with BCG alone or with BCG+control cells was comparable, while the response of cells cultured with BCG+suppressor cells was about 40% of the above two responses (Fig. 3a). The flow cytometric analysis showed that on average, 18% of the viable cells were anti-Tac positive when fresh cells were cultured with suppressor cells and BCG, whereas, 34% showed reactivity to antibody when cultured with BCG alone or BCG+irradiated control cells (Fig. 3b).

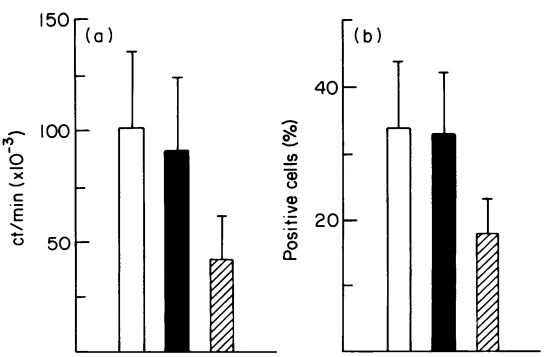

Fig. 3. Suppressor cells inhibit induction of, (a) IL-2 responsiveness, and (b) the induction of IL-2 receptors, on fresh cells. Fresh cells were cultured with BCG alone (□), BCG+irradiated control cells (■) or BCG+irradiated suppressor cells (▨) for 6 days. Viable cells were recovered by floatation on lymphoprep gradient and assayed, (a) for responsiveness to IL-2, and (b) for the percentage of positive cells reacting with anti-Tac antibody. Results are expressed as mean±s.e.m. of four different experiments from different subjects.

DISCUSSION

We have earlier demonstrated the *in vitro* induction of suppressor T cells from BCG-vaccinated healthy individuals. These suppressors had CD4 phenotype, were antigen specific and MHC restricted (Mustafa & Godal, 1983). In the experiments reported in this paper, we have studied the conditions optimal for their induction. In many other *in vitro* systems high doses of antigen (Uytdehaag, Heijnen & Ballieux, 1978; Morimoto *et al.*, 1983) or antigen given in the absence of adherent antigen-presenting cells (Feldman & Kontainen, 1976; Piccolella, Lombardi & Morelli, 1981) resulted in antigen specific suppressor-cell induction. However, in our studies the optimal conditions for antigenic stimulation of the cells in a proliferative response, i.e. optimal antigen and monocyte concentrations also resulted in maximum suppression. This may mean that precursors of all cell types are present in normal PBMC, and depending upon the mode of stimulation, a given type of effector cell will functionally predominate. Such a phenomenon has been demonstrated in mouse systems using *M. leprae* and BCG. Intradermal or subcutaneous immunization resulted in the development of cellular immunity, whereas, when bacilli were directly presented to the immune cells in circulation by the intravenous route, there was suppression of the immune response (Shepard *et al.*, 1982; Turcotte & Forget, 1983).

T cell proliferation is dependent upon the availability of IL-2 and the induction of IL-2 receptors (Cantrell & Smith, 1984). In addition to specific stimulus, IL-2 production by T cells requires IL-1 Larsson, Iscove & Coutinho, 1980; Smith *et al.*, 1980). In our system, BCG-induced suppressor cells did not suppress IL-1 production by adherent cells, but, affected IL-2 activity in the secondary culture. The possibility that decrease in IL-2 activity could have been due to soluble factors blocking IL-2 activity in the assay is remote because we could not demonstrate suppressor activity in the supernatants of suppressor cells + BCG (data not shown).

Recombinant IL-2 was directly added to the secondary cultures to monitor its effect on suppression. There was little effect at lower concentrations of IL-2, but at higher concentrations suppression was partially abrogated. However, the situation was complicated as at higher concentrations, IL-2 alone in the absence of antigenic stimulus was causing high background proliferation of cells (Table 3). Therefore, the partial abrogation of suppression observed could have been due to additional recruitment of either antigen-specific or non-specific cells.

The findings on Tac receptor bearing and IL-2 responding cells on day 6 of secondary cultures suggest that suppressor cells could have suppressed IL-2 receptor expression as well. However, since availability of IL-2 can regulate both proliferation and IL-2 receptor expression on T cells (Reem & Yeh, 1984), these results on day 6 do not provide the definite evidence for suppressor cells directly suppressing IL-2 receptor expression by fresh cells. It would have been more meaningful to measure IL-2 receptor expression at a time before commencement of proliferation, e.g. 24–48 h after stimulation, but this was not feasible as BCG-activated cells had $< 5\%$ Tac receptor bearing cells up to 48 h of stimulation (data not shown). The inability of recombinant IL-2 to abrogate suppression completely, is compatible with the possibility that suppressor cells might have a major effect at the level of IL-2 receptor expression.

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