

## Suppression of BCG cell wall induced delayed-type hypersensitivity by BCG pre-treatment

### I. INDUCTION OF ADHERENT SUPPRESSOR CELLS BY LIVE BCG INJECTION AND THEIR CHARACTERIZATION

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**Summary.** Previous injections of live *Bacillus Calmette-Guérin* (BCG) in mice produced a suppression of delayed-type hypersensitivity (DTH) induced by oil-treated BCG cell walls (CW). This phenomenon was analysed by the macrophage migration inhibition (MI) test in which peritoneal exudate cells (PEC) from live BCG-injected mice were mixed with PEC from BCG CW-immunized mice, with the result that the former cells suppressed the MI activity in the latter. We considered the MI test to be a reliable method for demonstrating the existence of suppressor cells induced by the injection of live BCG. Moreover, we found that the adherent cells of PEC possessed a suppressive effect which was retained even after treatment with either anti-mouse Ig or anti-brain associated  $\theta$  (BA $\theta$ ) antigen; that the PEC from mice injected with live BCG on at least the 12th day before cell harvesting showed the suppression; and that the suppression operated across the H-2 barrier.

#### INTRODUCTION

*Bacillus Calmette Guérin* (BCG) is used widely in immunological experiments and in tumour im-

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munotherapy as an effective immunopotentiator. Although the exact mechanism of BCG-mediated immunostimulation and its role in tumour immunotherapy are not understood completely, the association of BCG-stimulated macrophage activation with tumour and/or micro-organism resistance has been established.

On the other hand, there have been several reports indicating that live BCG produces suppressive effects on cell-mediated immunity. It has been shown, for example, that spleen cells from BCG-treated animals show a poor graft-*v.*-host reaction (Geffard & Orbach-Arbouys, 1976), that they cause the suppression of T-cell mitogen responsiveness in normal spleen cells (Florentin, Huchet, Bruley-Rosset, Halle-Pannenko & Mathé, 1976; Klimpel & Henney, 1978; Tarcotte, Lafleur & Labreche, 1978), and that they inhibit the primary cytotoxic responses *in vitro* (Klimpel & Henney, 1978). The above-mentioned reports have established that the adherent cells in the spleen elicited by BCG administration play an important role as immunoregulators.

In this investigation we have observed that DTH, which was estimated by footpad reactions in mice immunized with BCG cell walls (CW), was suppressed by previous injections of live BCG. There have been few reports on DTH suppression induced by BCG as analysed by use of macrophage migration inhibition

(MI) test. We analysed the delayed-type hypersensitivity (DTH) suppression using the MI test. In addition, we have observed that the adherent cells of peritoneal exudate cells (PEC) from mice injected with live BCG inhibited the MI activity of PEC from the mice immunized with BCG CW. Our findings suggest that the suppression of DTH is regulated by adherent cells induced by live BCG.

## MATERIALS AND METHODS

### *Animals*

The C3H/HeMs mice (C3H) were bred in our animal facility from breeding pairs supplied by the National Institute of Genetics, Mishima, Shizuoka Prefecture, Japan. The C3H/HeMs mice have *Car-2<sup>a</sup>* allele on chromosome 3, however, nineteen other markers including the *H-2* of these mice studied so far were the same as the original the C3H/He mice. The C57Bl/6 mice (B6) were obtained from the Shizuoka Laboratory Animal Agricultural Cooperation Association, Hamamatsu, Shizuoka. All mice were used at 4–8 weeks of age. The B6 mice were used only in the experiment in which mice having a major histocompatibility complex different from the C3H mice were required.

### *Antigens*

The BCG CW was kindly donated by Dr Ribi, Rocky Mountain Laboratory, NIH, Hamilton, Mont., and the purified protein derivatives (PPD) were obtained through the courtesy of Dr Brehmer, Robert Koch Institute, Berlin.

### *Immunization*

The BCG CW vaccine was prepared by the method of Brehmer, Anacker & Ribi (1968). The typical procedure used in this study have been described in our previous paper (Yamamoto & Kakinuma, 1978). Vaccine (0.2 ml) containing 300 µg of BCG CW was injected subcutaneously into the mice.

### *BCG*

The BCG (Japanese strain) was cultured in 7H9 media at 37° for 7–10 days. After harvesting of the cultures by centrifuge and washing them once with 7H9 media without enrichment, the BCG was resuspended in the 7H9 media and maintained at –70° until later use. This BCG suspension contained an approximately  $5 \times 10^8$  viable colony forming unit/ml. For prepa-

ration of the heat-killed BCG, freshly thawed BCG was suspended in saline after one washing with saline, and the suspension was heated at 100° for 30 min in Köch's steam bath. Two-tenths millilitre of live and/or heat-killed BCG (0.2 ml) were injected intravenously (i.v.) or intraperitoneally (i.p.) into each group of mice.

### *Footpad test for DTH*

To determine the DTH, the footpad test was performed 4 weeks after the immunization with BCG CW; the procedures have been detailed in an earlier study (Yamamoto & Kakinuma, 1978).

### *Antiserum preparation*

Anti-BA $\theta$  serum was prepared in rabbits according to the method of Golub (1971). To obtain the anti-mouse Ig, an IgG fraction of pooled normal C3H mice serum was prepared by two successive precipitations with sodium sulphate to final concentration of 18% and 16% respectively, and the resultant protein was passed through a DEAE cellulose column in a 0.04 M phosphate buffer, pH 8.0 (Spring & Nisonoff, 1974). The antisera against mouse IgG were obtained by immunizing the rabbits with mouse IgG which had been incorporated in Freund's complete adjuvant, after which they were inactivated at 56° for 30 min before use, and then absorbed twice with an equal volume of packed mouse erythrocytes at room temperature for 1 h. The anti-BA $\theta$  was also absorbed with mouse liver cells.

### *MI test*

Basically, the MI test was performed as described previously (Yamamoto, Anacker & Ribi, 1970). The PEC used in the MI test were collected in Hanks's balanced salt solution on the 3rd day after i.p. injection of 2 ml of 12% casein sodium (Nakarai Chemicals Ltd, Kyoto) saline. The cells were washed three times in the Hanks's balanced salt solution and then resuspended to a concentration of 10% in Eagle's minimum essential medium (MEM), to which were added 100 u penicillin, 100 µg streptomycin, and 15% foetal calf serum (Gibco, Berkeley, Calif.) per ml. The peritoneal cell suspension was centrifuged in capillaries at 200g, and the portion of capillary containing the packed cells was then attached with silicone to the bottom cover slip of Sykes–Moore tissue culture chambers. The chambers were then filled with the above-mentioned MEM with or without 50 µg of PPD/ml, and incubated at 37° for 24 h.

The areas of cell migration from the open end of the capillary were traced on paper after projection and enlargement, and then measured with a planimeter. The percentage of migration was calculated by dividing the test cells' area of migration by the area of migration of control cells, and multiplying by 100.

The inhibitory effects of PEC from live or heat-killed BCG-injected mice (L-BCG-PEC or K-BCG-PEC) on the MI activity were tested in a 1:1 ratio mixture with MI positive PEC which had been harvested from BCG CW-immunized mice (effector PEC).

**RESULTS**

**The effect of pre-treatment with BCG on the development of DTH**

The C3H mice were injected i.v. or i.p. with 10<sup>8</sup> live or heat-killed BCG 3 weeks before the immunization with BCG CW, and the footpad test was done 4 weeks after the immunization with BCG CW. As shown in Fig. 1, the live BCG-treated mice failed to generate any significant response; on the other hand, the mice injected i.p. with heat-killed BCG showed a strong response which was comparable to that of the control

mice, while the mice injected i.v. with the heat-killed BCG did not demonstrate any response at all.

**The specificity of suppressive effect of BCG pretreatment on the development of DTH**

We next examined whether or not the suppression of DTH development induced by the BCG pretreatment was antigen specific.

A water-in-oil emulsion of 500 µg of acetylated bovine serum albumin (AcBSA) and 300 µg of N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) were used for immunization and the footpad reaction was examined with 35 µg of AcBSA. As shown in Table 1,

**Table 1.** Suppression of DTH induced by AcBSA plus MDP in live BCG-pretreated mice

	Live BCG pretreatment*	Non-treated control
Footpad reaction (mm)	0.14 ± 0.05	0.35 ± 0.04

*P* < 0.001.

\* The C3H mice were injected i.v. with 10<sup>8</sup> live BCG 3 weeks before the immunization with AcBSA plus MDP.

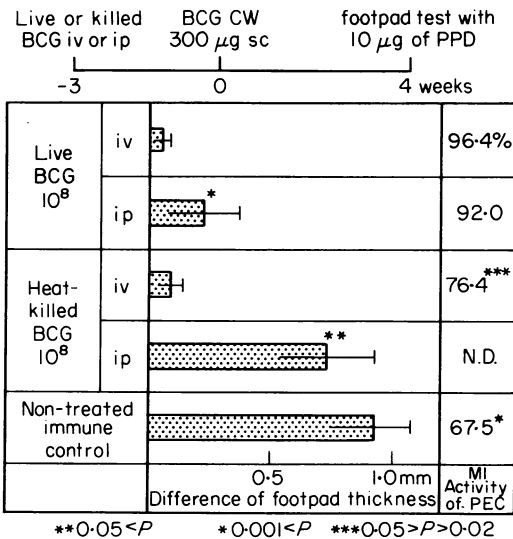
the live BCG-pretreated mice failed to generate a significant footpad response with the AcBSA, as compared with the non-treated control mice, which suggested that non-antigen specific suppression was produced by the live BCG injection.

**MI activity of PEC from the BCG-pretreated mice which failed to generate a DTH response**

The unresponsive state of DTH in the BCG-pretreated mice was examined *in vitro* using the MI test, which was considered to be an *in vitro* criterion of DTH. As also shown in Fig. 1, no MI activity of PEC from the live BCG-pretreated mice was observed. The *in vitro* and *in vivo* findings showed good correlation, however, MI activity of the PEC from the mice which had received i.v. injections of heat-killed BCG was observed despite of the absence of DTH.

**MI activity of a PEC mixture from effector PEC with L-BCG-PEC or K-BCG-PEC**

We analysed *in vitro* the mechanisms of DTH suppres-



**Figure 1.** DTH responses in C3H mice sensitized with BCG CW. The C3H mice were injected i.v. or i.p. with the live or heat-killed BCG 3 weeks before the immunization with BCG CW. Footpad thickness was measured 4 weeks after the immunization with BCG CW. MI activity of PEC was estimated by the percentage migration of the cells. ND: not done.

**Table 2.** Inhibitory effect of PEC from live BCG-injected mice on MI activity of PEC from BCG CW-immunized mice

PEC from	Mixed with PEC from	Presence of PPD	Areas of migration (cm <sup>2</sup> )	MI activity (%)
Mice† immunized subcutaneously with 300 µg of oil-treated BCG CW	—	{ (-)	3.87 ± 0.05	63.8*
		{ (+)	2.47 ± 0.44	
Live BCG-injected mice‡	Normal mice	{ (-)	2.55 ± 0.25	55.6*
		{ (+)	1.42 ± 0.25	
	Live BCG-injected mice‡	{ (-)	2.17 ± 0.26	124.1
		{ (+)	2.7 ± 0.1	
Killed BCG-injected mice‡	Killed BCG-injected mice‡	{ (-)	2.55 ± 0.17	62.7*
		{ (+)	1.6 ± 0.54	
Live BCG-injected mice‡		{ (-)	1.82 ± 0.12	85.1**
		{ (+)	1.55 ± 0.20	
Killed BCG-injected mice‡		{ (-)	1.70 ± 0.11	112.3
		{ (+)	1.91 ± 0.28	

\*  $P < 0.001$ .\*\*  $0.1 > P > 0.05$ .

† These mice were immunized 4 weeks before cell harvest.

‡ These mice were injected intravenously with  $10^8$  live or heat-killed BCG 3 weeks before cell harvest.Areas of migration are expressed as  $\pm$  SD.

This table shows the representative results of an experiment performed at least three times which yielded similar results.

sion due to BCG intravenous administrations. The L-BCG-PEC and K-BCG-PEC were obtained 3 weeks after the live and heat-killed BCG i.v. injections while the effector PEC were obtained 4 weeks after the BCG CW injection. An equal volume of L- or K-BCG-PEC and effector PEC was mixed and their MI activities were measured. In addition, a mixture of effector PEC with PEC from normal mice (N-PEC), instead of L- or K-BCG-PEC, was prepared as a control, and its MI activity was measured. As shown in Table 2, there was

no MI activity observed when the L-BCG-PEC were mixed, which suggested that the MI activity of effector PEC was suppressed by L-BCG-PEC. One hypothesis for this suppression was that the insensitivity of L-BCG-PEC to the migration inhibitory factor (MIF) produced with the effector PEC did not inhibit the migration of the L-BCG-PEC, and that it masked the migration inhibition of the effector PEC. We attempted to exclude this hypothesis by examining the L-BCG-PEC for its sensitivity to MIF, which exists in

**Table 3.** MIF sensitivity of PEC from mice† injected i.v. with live BCG

Serum added to MI-test medium	Area of migration of PEC (cm <sup>2</sup> )	Percentage of migration
Desensitized serum‡ containing MIF	1.97 ± 0.38	59.3*
Immune control serum§	3.25 ± 0.30	

\*  $0.001 < P < 0.01$ .† These mice were injected intravenously with  $10^8$  live BCG 3 weeks before cell harvest.

‡ The serum was obtained from BCG CW-immunized mice which had been injected i.v. with BCG protoplasm 1 day before serum harvest.

§ The serum was obtained from BCG CW-immunized mice without injecting BCG protoplasm.

the serum of BCG CW-immunized mice which had been desensitized with BCG protoplasm (Yamamoto & Takahashi, 1971). As shown in Table 3, the migration of L-BCG-PEC was obviously inhibited by the migration inhibitory serum, indicating that the L-BCG-PEC were not insensitive to MIF. Thus the lack of migration inhibition in the mixed culture could not be attributed to the L-BCG-PEC's resistance to MIF.

On the other hand, the K-BCG-PEC in the previous experiment did not show these suppressive results of the MI test (Fig. 1).

#### Characterization of suppressor cells from L-BCG-PEC

In order to characterize the cell type responsible for the suppression of MI activity, the L-BCG-PEC were placed on plastic petri dishes and incubated at 37° for 2 h to separate the adherent from the non-adherent cells. We also examined the suppressive effects of the adherent and non-adherent cells on the MI activity of the effector PEC. Table 4 shows that the adherent cells in the L-BCG-PEC evidently suppressed the MI activity of the effector PEC. Furthermore, the suppressive activity of the adherent cells in the L-BCG-PEC was fully retained after their incubation with either anti-mouse Ig or anti-BA $\theta$  in the presence of guinea-pig complement (Table 5). In immunofluorescence tests using FITC-anti-mouse Ig and FITC-anti-BA $\theta$ , it was confirmed that treatment with anti-mouse Ig plus complement or anti-BA $\theta$  plus complement was effective for the elimination of B and T cells, respectively.

#### The time course of development of suppressive effect of L-BCG-PEC on MI activity

In order to determine the time course of development of the suppressor cells on the MI activity, L-BCG-PEC were obtained at 3, 12 and 21 days after the injections. Each L-BCG-PEC was mixed with effector PEC and then assayed for MI activity. Table 6 shows that a suppression of MI activity in the mixture occurred when 12- and 21-day-old L-BCG-PEC were mixed.

#### Suppression occurring across the H-2 barrier

The following experiment was done in order to determine the presence of any H-2 restriction on the suppressive effect of L-BCG-PEC. The PEC from C3H mice injected with BCG CW were mixed with the N-PEC or L-BCG-PEC from B6 mice. In the same manner, the PEC from the B6 mice were mixed with the PEC from the C3H mice, and the mixtures were assayed for their MI activity. As shown in Table 7, the L-BCG-PEC from the B6 mice (*H-2<sup>b</sup>*) clearly suppressed the MI activity of the effector PEC from the C3H mice (*H-2<sup>k</sup>*). Similarly, the L-BCG-PEC from the C3H mice also suppressed the MI activity of the effector PEC from the B6 mice. These findings showed that the suppression of L-BCG-PEC on MI activity could take place across the H-2 barriers.

## DISCUSSION

In the present investigation we have established that

**Table 4.** Inhibitory effect of adherent PEC from live BCG-injected mice on MI activity of PEC from BCG CW-immunized mice

PEC from	Mixed with	MI activity (%)
Mice† immunized subcutaneously with 300 $\mu$ g of oil-treated BCG CW	PEC from normal mice	64.7*
	PEC from live BCG-injected mice‡	110.3
	Adherent PEC from live BCG-injected mice	115.7
	Non-adherent PEC from live BCG-injected mice	73.9**

\*  $P < 0.001$ .

\*\*  $0.01 < P < 0.02$ .

† These mice were immunized 4 weeks before cell harvest.

‡ These mice were injected intravenously with  $10^8$  live BCG 3 weeks before cell harvest.

**Table 5.** Inhibitory effect of adherent PEC from live BCG-injected mice on MI activity of PEC from BCG CW-immunized mice after various treatments

PEC from	Mixed with	MI activity (%)
Mice† immunized subcutaneously with 300 µg of oil-treated BCG CW	PEC from normal mice	64.7*
	Adherent PEC from live BCG-injected mice‡	112.8
	Anti-Ig treated adherent PEC from live BCG-injected mice	95.4
	Anti-BAθ treated adherent PEC from live BCG-injected mice	92.1

\*  $P < 0.001$ .

† These mice were immunized 4 weeks before cell harvest.

‡ These mice were injected intravenously with  $10^8$  live BCG 3 weeks before cell harvest.

pretreatment with live BCG generates a strong suppressive effect on the development of DTH with BCG CW in mice, and that the induction of the suppression is non-antigen specific. Our observation appears to agree with that of Lamoureaux & Poisson (1974), who found that guinea-pigs undergoing chronic BCG therapy showed non-specific unresponsiveness of cell-mediated immunity. However, the mechanisms of the unresponsiveness of cell-mediated immunity have been unclear up to now. We have attempted to clarify the mechanism of DTH suppression by using an MI assay in this study, since the BCG CW-immunized mice which had been previously injected with live BCG showed neither a footpad reaction nor any MI activity of the PEC (Fig. 1). When L-BCG-PEC were mixed with effector PEC, there was no observable MI activity (Table 2), which suggested strongly that the suppressor cells in the L-BCG-PEC inhibited the MI activity of the effector PEC. We were able to demon-

strate clearly that the migration of L-BCG-PEC was inhibited by the migration inhibitory serum (Table 3), which fairly excluded the possibility that the L-BCG-PEC in the mixed culture migrated normally, giving the appearance that the suppression resulted from the L-BCG-PEC's resistance to MIF. Although the possibility remains that the sensitivity of L-BCG-PEC to MIF produced *in vitro* is different from that to MIF in serum from desensitized mice, it is clear that the L-BCG-PEC were not insensitive to the MIF. We are now investigating the possibility that the suppressor cells acted on the T cells of the effector PEC to prevent them from elaborating MIF.

We next examined the character of the supposed suppressor cells in the L-BCG-PEC which affected the MI activity of the effector PEC. The suppressive function of L-BCG-PEC was found in the adherent cell population, and its suppressive function was retained after treatment with either anti-mouse Ig or anti-BAθ

**Table 6.** Time course of development of suppressor cells after live BCG injection

PEC from	Mixed with	MI activity (%)
Mice† immunized subcutaneously with 300 µg of oil-treated BCG CW	N-PEC	83.7*
	3 day-PEC‡	71.7*
	12 day-PEC‡	112.1
	21 day-PEC‡	105.6

§  $0.001 < P < 0.01$ .

† These mice were immunized 4 weeks before cell harvest.

‡ PEC from mice intravenously injected with live BCG 3, 12 and 21 days before cell harvest, respectively.

**Table 7.** Inhibitory effect of PEC from live BCG-injected mice on MI activity of PEC from BCG CW-immunized allogeneic mice

PEC from BCG CW sensitized mice†	Mixed with PEC from	MI activity (%)
C3H/HeMs (C3H)	Non-treated C3H	70.2*
	Live BCG-injected C3H‡	105.1
	Non-treated B6	69.2*
	Live BCG-injected B6‡	109.2
C57Bl/6 (B6)	Non-treated B6	74.0*
	Live BCG-injected B6‡	106.0
	Non-treated C3H	64.4*
	Live BCG-injected C3H‡	110.4

§ 0.05 >  $P$  > 0.02.

† These mice were immunized 4 weeks before cell harvest.

‡ These mice were injected intravenously with  $10^8$  live BCG 3 weeks before cell harvest.

with guinea-pig complement. We concluded, therefore, that the suppressor cells may belong to the macrophage compartment. We showed that the suppressor cells suppressed the MI activity of effector PEC obtained from syngeneic and allogeneic mice, which suggested that the suppressor cells operated without H-2 restrictions (Table 7).

Lagrange & Mackaness (1975) and Mackaness, Lagrange & Ishibashi (1974), however, have reported that pretreatment with live BCG markedly enhanced the DTH response to sheep red blood cells (SRBC). It remains unclear why BCG modulated the DTH responses induced with SRBC and BCG CW in different ways. The suppressor cells detected in this investigation may be similar to the macrophages in the spleen of BCG-injected mice which have been studied by several investigators. The BCG-induced suppressor macrophages in the spleen have been found to affect both the T-cell proliferation (Florentin *et al.*, 1976; Klimpel & Henney, 1978; Tarcotte *et al.*, 1978) and the cytotoxic T-cell generation (Klimpel & Henney, 1978). Recently, Bennet, Rao & Mitchell (1978) have reported that such suppressor macrophages exist in normal bone marrow, and that they are stimulated by i.v. injections of live BCG.

It has been established that, in addition to BCG, *Corynebacterium parvum* induces suppressor macrophages in mice which show inhibited mitogen responses of normal syngeneic spleen T cells (Scott, 1972; Kirchner, Holden & Herberman 1975). In human diseases, the adherent cells which appear to be the monocyte/macrophage type have been regarded as

the immunoregulatory cells in Hodgkin's disease (Twomey, Laughter, Farrow & Douglas 1975; Goodwin, Messner, Bankhurst, Peak, Saihke & Williams, 1977), multiple myeloma (Broder, Humphrey, Durm, Blackman, Meade, Goldman, Strober & Waldman, 1975), SLE (Markenson, Morgan, Lockshin, Joachim and Winfield, 1978), disseminated fungal infection (Stobo, 1977), and pulmonary tuberculosis (Ellner, 1978).

Regarding the injection of heat-killed BCG, the footpad reaction of the BCG CW-immunized mice was suppressed by pre-treatment with heat-killed BCG i.v. but not with i.p. injection. The MI activity of PEC from mice which were i.v. pre-treated with heat-killed BCG was not depressed, and the adherent suppressor cells were not induced in the peritoneal cavity. However, when effector PEC were mixed with the non-adherent spleen cells of mice injected with heat-killed BCG, no MI activity was detected (Kato, unpublished), which suggests the existence of non-adherent suppressor cells in the spleen. These findings support the possibility that the unresponsiveness of DTH by i.v. injection of live BCG and heat-killed BCG is caused by different mechanisms. Although we have not completely excluded the possibility that the phenomenon observed in this study may be simply a manifestation of the growth of live BCG in the PEC, it appears clear that there is no effect of the growth of live BCG in the PEC on its MI activity, because no difference was found in the migration areas of N-PEC and L-BCG-PEC ( $P > 0.05$ ) when they were mixed with effector PEC in the absence of PPD (Table 2).

We are continuing the investigation of DTH suppression by i.v. injection of heat-killed BCG, and the relationship between the BCG-induced suppressor cells and the BCG CW-sensitized T cells.

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