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

II. INDUCTION OF SUPPRESSOR T CELLS BY HEAT-KILLED BCG INJECTION

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Summary. Previous intravenous (i.v.) injection of heat-killed *Bacillus Calmette-Guérin* (BCG) in mice produced a suppression of delayed-type hypersensitivity (DTH) induced with oil-treated BCG cell walls. This phenomenon was analysed by the macrophage migration inhibition (MI) test in which non-adherent spleen cells from mice which had been injected with heat-killed BCG (K-Non-ad. cells) were mixed with peritoneal exudate cells from BCG cell wall-immunized mice (effector-PEC). The test showed that the K-Non-ad. cells suppressed the MI activity in the effector-PEC, which indicated that the suppressor cells were induced in the spleen by the heat-killed BCG injections. Moreover, the suppressive effect of the K-Non-ad. cells disappeared after treatment with anti-brain associated θ serum (BAθ) and guinea-pig complement, and operated across the H-2 barrier. The suppressor cells inhibited the production or release of the migration inhibition factor from the BCG cell wall-sensitized cells in the presence of the specific antigen, purified protein derivatives (PPD). It was concluded that the injections of heat-killed BCG produced antigen non-specific suppressor T cells in DTH suppression.

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INTRODUCTION

In a previous report, the authors (Kato, Yamamoto, Kakinuma, Ishihara & Azuma, 1981) showed that delayed-type hypersensitivity (DTH) estimated by footpad reactions in mice immunized with *Bacillus Calmette-Guérin* (BCG) cell walls was suppressed by previous intravenous (i.v.) injections of a large dose of live or heat-killed BCG. In the case of the live BCG, the macrophage migration inhibition (MI) test revealed a close correlation between the *in vitro* (MI test) and the *in vivo* findings (footpad reaction) concerned with DTH suppression and suggested that macrophage (-like) cells were involved in the suppression. However, in the case of the heat-killed BCG, MI activity of peritoneal exudate cells (PEC) was observed in spite of the absence of footpad reactions in the treated mice. This finding suggested that no suppressor cells were induced in the peritoneal cavity by the heat-killed BCG injection.

Several reports indicating that suppressor cells resident in the spleen play a regulatory role in cell-mediated immunity have appeared in the literature (Ha & Waksman, 1973; Phamuphak, Moorhead & Claman, 1974; Sy, Miller, Kowach & Claman; 1977; Greene, Sugimoto & Benacerraf, 1978; Tagart, Thomas & Asherson, 1978; Kojima & Egashira, 1979; Nakamura & Tokunaga, 1980; Watson & Collins, 1980). In the present study, the involvement of splenic

cells in the DTH suppression induced by heat-killed BCG injection was investigated by the MI test.

The results showed that the Sephadex G-10 column passed non-adherent splenic cells from mice injected with heat-killed BCG suppressed the MI activity of PEC from BCG cell wall-immunized mice (effector-PEC), which suggested that these suppressor cells were involved in the suppression of DTH induced by BCG cell wall immunization.

MATERIALS AND METHODS

Animals

Six to eight-week-old C3H/HeMs mice (C3H) of either sex were used. C57BL/6 mice (B6) were used in the experiment which required mice showing a histocompatibility complex which was different from C3H mice.

Antigen, BCG, immunization and footpad test for DTH

The BCG cell walls and purified protein derivative (PPD) were kindly donated by Dr Ribi, Rocky Mountain Laboratory, N.I.H., Hamilton, Montana and by Dr Brehmer, Robert Koch Institute, Berlin. BCG cell wall vaccine was prepared by the method of Brehmer, Anacker & Ribi (1968). Subcutaneous injections of 0·2 ml of vaccine containing 300 μg BCG cell walls, and of 1 mg of heat-killed *Listeria* monocytogenes, EGD, in a water-in-oil emulsion (Freund's incomplete type) were administered to the mice. The footpad test was performed 9 days after the immunization with *Listeria* to determine DTH. For this test, 10 μg of *Listeria* protoplasm in 0·05 ml saline were injected intradermally (i.d.) into the left rear footpad, and as a control, 0·05 ml of saline was injected into the right footpad. Footpad thickness was measured 24 hr later by a dial-gauge caliper (Schnell-taster, Kröplin Schwchtern), and the difference between the *Listeria* protoplasm- and the saline-injected rear footpads was expressed as the footpad reaction. BCG (Japanese strain) and heat-killed BCG were prepared as described previously (Kato *et al.*, 1981); About 1 mg of heat-killed BCG was administered intravenously to the mice.

Antiserum preparation

Anti-BAθ serum and anti-mouse Ig serum were prepared as described in our previous paper (Kato *et al.*, 1981).

Preparation of non-adherent cells from spleen cells

After erythrocytes from the mouse spleen cells had been removed by 0·83% NH_4Cl-Tris buffer, the cells were washed three times with Hanks's balanced salt solution and resuspended in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) containing 10% foetal calf serum (GIBCO, Grand Island, N.Y.). The non-adherent cells were separated from the spleen cells by applying the cells to a Sephadex G-10 column to deplete the macrophages. Briefly, the spleen cells were suspended in RPMI 1640 medium with 10% foetal calf serum at a concentration of 1×10^8/ml, and 1 ml of the suspension was applied to the Sephadex G-10 in a 10 ml syringe and incubated for 45 min at 37°. The non-adherent cells were eluted with warm RPMI 1640 medium containing 10% foetal calf serum.

Migration inhibition test

To examine the inhibitory effect of the non-adherent spleen cells from the heat-killed or live BCG injected mice (K-Non-ad. or L-Non-ad. cells) on the MI activity of PEC from mice which had been immunized with BCG cell walls (effector-PEC), both types of cells were mixed at a ratio of 1:9 and the MI activity of the mixture was measured. The MI activity was expressed by the migration inhibition index calculated from the results of the MI test which was performed as described in the previous paper (Kato *et al.*, 1981).

Preparation of culture supernatant fluid

As described above, the non-adherent cells in PEC were obtained from mice which had been immunized with 300 μg of BCG cell wall vaccine 4 weeks earlier. These cells 3×10^6 and the same number of K-Non-ad. cells or non-adherent spleen cells from normal mice (N-Non-ad. cells) were cultured in 4 ml of RPMI 1640 medium supplemented with 10% foetal calf serum. After incubation for 24 hr at 37° in a 5% CO_2 humidified atmosphere, the culture supernatant fluids obtained by centrifugation were dialysed against distilled water and lyophilized. The lyophilized material was then dissolved into 4 ml of the culture medium used for the MI test as the original supernatant fluid.

RESULTS

Specificity of suppressive effect of heat-killed BCG pretreatment on the development of DTH

We examined whether the suppression of BCG cell-wall DTH by the heat-killed BCG pretreatment was

Table 1. Inhibitory effect of non-adherent spleen cells from heat-killed BCG injected mice on MI activity of PEC from BCG cell wall immunized mice

Effector	Mixed with non-adherent spleen cells* from	MI activity (%)	
PEC from BCG cell walls immunized mice† (effector PEC)	Non-treated mice	59·0	$(0{\cdot}001 < P < 0{\cdot}01)$
	Heat-killed BCG-injected mice‡	104·8	
	Live BCG-injected mice§	68·3	$(0{\cdot}001 < P < 0{\cdot}01)$

* These cells were mixed with effector PEC in a ratio of 1:9.
† Immunized with 300 μg of BCG cell wall vaccine 4 weeks before cell harvest.
‡ § Injected i.v. with 10^8 heat-killed BCG or 10^8 live BCG 3 weeks before cell harvest, respectively.

antigen specific. For this test, 1 mg of *Listeria* in Freund's incomplete emulsion was injected subcutaneously (s.c.) into mice which had been i.v. pretreated with 10^8 heat-killed BCG 3 weeks before. Nine days later these mice were examined for their footpad reactions using 10 μg of *Listeria*-protoplasm. The heat-killed BCG pretreated mice failed to show a significant footpad response ($0{\cdot}18 \pm 0{\cdot}08$ mm) as compared with the non-treated control mice ($0{\cdot}42 \pm 0{\cdot}08$ mm; $0{\cdot}001 < P < 0{\cdot}01$), suggesting that antigen non-specific suppression was produced by the heat-killed BCG injection.

MI activity of mixture of effector PEC with K-Non-ad. cells

From the results of our previous studies (Kato *et al.*, 1981), it appeared that the suppressor cells against effector PEC induced by pretreatment with heat-killed BCG did not migrate into the peritoneal cavity. Thus we next examined the possibility that the suppressor cells occurred in the spleen cells, especially in the K-Non-ad. population.

K-Non-ad. cells from mice which had been injected with about 10^8 heat-killed BCG 3 weeks earlier were mixed with effector PEC from mice which had been immunized with 300 μg of BCG cell walls 4 weeks earlier and their MI activity was measured. As controls, N-Non-ad. cells, Sephadex G-10 passed cells from normal mouse spleens and L-Non-ad. cells from mice which had been injected with about 10^8 live BCG 3 weeks earlier were examined for their suppressive effect on the MI activity of effector PEC.

No MI activity of the effector PEC and K-Non-ad. cells mixture was observed, suggesting that the MI activity of effector PEC was suppressed by K-Non-ad. cells while N-Non-ad. and L-Non-ad. cells had no effect (Table 1).

Characterization of suppressor cells from K-Non-ad. population

In order to characterize the cell type responsible for the suppression, K-Non-ad. cells were treated with either anti-mouse Ig serum with 3 mM NaN_3 or anti-BAθ serum in the presence of guinea-pig complement. The results showed that the latter destroyed the suppressive effect of K-Non-ad. cells on the MI activity of effector PEC (Table 2). Furthermore, the immunofluorescence test using FITC-anti-mouse Ig and FITC-anti-BAθ confirmed that treatment with anti-mouse Ig plus complement eliminated over 98% of the B cells and anti-BAθ plus complement eliminated over 99% of the T cells.

Suppression occurring across the H-2 barrier

We next examined whether the suppressive effect of K-Non-ad. cells was restricted to syngeneic effector PEC. The effector PEC from C3H mice were mixed with K-Non-ad. cells or N-Non-ad. cells from B6 mice, and the mixture was assayed for MI activity. Results showed that the K-Non-ad. cells from the B6 mice ($H\text{-}2^b$) clearly suppressed the MI activity of the effector PEC from the C3H mice ($H\text{-}2^k$), Table 3.

Inhibition of macrophage migration inhibitory factor (MIF) production or release from BCG cell-wall sensitized non-adherent cells in the presence of K-Non-ad. cells

The lack of MI activity in the mixture of effector PEC

Table 2. Suppression of MI activity of PEC from BCG cell wall-immunized mice* with non-adherent spleen cells from mice† i.v. injected with heat-killed BCG

Effector PEC from BCG cell wall-immunized mice plus‡	MI activity (%)	
Normal non-adherent spleen cells	51·4	($P < 0{\cdot}001$)
Heat-killed BCG non-adherent spleen cells	113·5	
Anti-IgG serum-treated heat-killed BCG non-adherent spleen cells	90·4	($0{\cdot}05 < P$)
Anti-BAθ serum-treated heat-killed BCG non-adherent spleen cells	60·5	($0{\cdot}01 > P > 0{\cdot}001$)

* Immunized with 300 μg of BCG cell walls 4 weeks before cell harvest.
† Intravenously injected with 10^8 heat-killed BCG 3 weeks before cell harvest.
‡ Non-adherent cells to be tested were mixed with effector PEC in a ratio of 1:9.

Table 3. Inhibitory effect of non-adherent spleen cells from heat-killed BCG-injected mice* on MI activity of PEC from BCG cell wall-immunized C3H/HeMs mice,† which operated across H-2 barrier.

	Added non-adherent spleen cells‡ from	MI activity (%)	
C57Bl/6	Normal mice	69·9	($0{\cdot}01 > P > 0{\cdot}001$)
	Heat-killed BCG-injected mice	123·7	
C3H/HeMs	Normal mice	56·8	($0{\cdot}05 > P > 0{\cdot}02$)
	Heat-killed BCG-injected mice	98·1	

* Intravenously injected with 10^8 heat-killed BCG 3 weeks before cell harvest.
† C3H/HeMs mice were immunized with 300 μg of BCG cell walls 4 weeks before cell harvest.
‡ These cells were mixed with PEC from BCG cell walls immunized C3H/HeMs mice in a ratio of 1:9.

Table 4. Inhibitory effect of non-adherent spleen cells from heat-killed BCG-treated mice on production or release of MIF from BCG cell wall-sensitized PEC in the presence of specific antigen, PPD

Supernatant fluids of mixed culture of effector PEC† with	Presence of PPD in culture	Concentrations of supernatant fluids	Migration areas of indicator cells§ (cm^2)
Normal non-adherent spleen cells	+	1:1	$2{\cdot}27 \pm 0{\cdot}40$*
	+	1:3	$2{\cdot}4 \pm 0{\cdot}14$
	+	1:7	$2{\cdot}22 \pm 0{\cdot}13$
	−	1:1	$3{\cdot}27 \pm 0{\cdot}33$*
	−	1:3	$2{\cdot}95 \pm 0{\cdot}38$
	−	1:7	$3{\cdot}27 \pm 0{\cdot}26$
Non-adherent spleen cells from heat-killed BCG-treated mice‡	+	1:1	$2{\cdot}80 \pm 0{\cdot}2$**
	+	1:3	$2{\cdot}82 \pm 0{\cdot}09$
	+	1:7	$3{\cdot}3 \pm 0{\cdot}33$
	−	1:1	$3{\cdot}0 \pm 0{\cdot}40$**
	−	1:3	$2{\cdot}75 \pm 0{\cdot}74$
	−	1:7	$3{\cdot}45 \pm 0{\cdot}28$

† The effector PECs were obtained from mice immunized with BCG cell walls 4 weeks after immunization.
‡ Intravenously injected with 10^8 heat-killed BCG 3 weeks before harvest.
§ The indicator cells were from normal mouse PEC. Differences in migration areas were significant in each corresponding concentration pair of normal non-adherent spleen cell groups (*$P < 0{\cdot}001$) but not in the heat-killed BCG spleen cell group (**$P > 0{\cdot}05$).

and K-Non-ad. cells suggested that the K-Non-ad. cells inhibited the production or the release of MIF from effector PEC in the presence of the specific antigen, PPD. This possibility was examined further by assaying the culture supernatant of the BCG cell wall sensitized non-adherent cells and the K-Non-ad. cells (K-sup.) mixture in the presence of PPD for their MIF activity using normal mouse PEC as indicator cells. As controls, culture supernatant fluids from the mixture of BCG cell wall sensitized non-adherent cells and N-Non-ad. cells (N-sup.) were used. By the same procedure as that used in the MI test, the migration area of indicator cells was measured in the presence of K-sup. or N-sup. diluted with the MI test medium in ratio of 1:1, 1:3 and 1:7, respectively. Table 4 shows that the migration areas were significantly decreased only by the N-sup. containing PPD as compared with those of the other three groups (K-sup. with PPD, N-sup. without PPD and K-sup. without PPD). This finding indicated the absence of MIF in the K-sup. with PPD, and suggested that the K-Non-ad. cells inhibited the BCG cell wall-sensitized non-adherent cells from producing or releasing MIF in the presence of PPD.

DISCUSSION

In this study, we have attempted to clarify the mechanism of suppression of BCG cell wall-induced DTH by previous i.v. injections of heat-killed BCG in mice. Earlier, we reported a similar DTH suppression which was induced by live BCG pretreatment and it appeared to be closely related to suppressor macrophage(-like cells) found in the peritoneal cavity, even though the pretreatment with heat-killed BCG did not induce such suppressive cells in the peritoneal cavity (Kato *et al.*, 1981).

Recently, several reports have indicated that splenic cells, particularly T cells, are involved in the suppression of cell-mediated immunity (Sy *et al.*, 1977; Greene *et al.*, 1978; Tagart *et al.*, 1978; Kojima & Egashira, 1979; Nakamura & Tokunaga, 1979; Watson & Collins, 1980). In our work, we attempted to isolate the candidate responsible for the DTH suppression from splenic cells of mice which had been injected with heat-killed BCG. Spleen cells from heat-killed BCG i.v. injected mice were passed on a Sephadex G-10 column and the resulting non-adherent cells (K-Non-ad. cells) were assayed for their suppressive activity against the MI activity of effector PEC. The results showed that the MI activity of effector PEC was suppressed by means of the K-Non-ad. cells (Table 1), suggesting the presence of suppressor cells for MI activity in the K-Non-ad. cells. On the other hand, no suppressor cells for MI activity in the L-Non-ad. cells were detected.

Continuing our investigation, we next attempted to characterize the K-Non-ad. suppressor cells. Here our test showed that the suppressive function of the K-Non-ad. cells became depleted after treatment with anti-BAθ serum and guinea-pig complement but not after treatment with anti-mouse Ig serum and guinea-pig complement. We concluded, therefore, that the suppressor cells are probably T cells.

In another test, there was an absence of MIF in the culture supernatant from the mixture of effector PEC with the suppressor cells in the presence of specific antigen, PPD (Table 4). This absence of MIF in the supernatants of the PPD-stimulated cultures containing the suppressor cells appeared to be due to the inhibition of MIF production or MIF release, although absorption or inactivation of MIF, or interference with the assay for detection of MIF cannot be ruled out.

We showed that the suppressor cells inhibited MI activity of effector PEC from allogeneic C3H mice, which suggested that the suppressor cells operated without H-2 restriction (Table 3).

Finally, we observed that pretreatment with heat-killed BCG suppressed the DTH induced with *Listeria*, which means that this suppression was not antigen-specific. There are many reports concerning the antigen-specific or antigen non-specific suppressor cells involved in cell-mediated immunity. As for non-antigen specific suppression, studies have shown that suppressor macrophage(-like cells) such as those induced by live BCG (Klimpel & Henney, 1978; Tarcotte, Lafleur & Labreche, 1978; Bennett, Rao & Mitchell, 1978; Orbach-Arbouys & Castes, 1980; Kato *et al.*, 1981), *Corynebacterium parvum* (Scott, 1972; Kichner, Holden & Herberman, 1975), *Shistosoma mansoni* (Coulis, Lewert & Fitch, 1978) and even non-antigenic adjuvant (Hanna, Blanc & Nelken, 1980) participate in the suppression. Recently, it was reported that suppressor T cells could be produced by BCG (Geffard & Orbach-Arbouys, 1976) and *Trypanosoma curzi* (Ramos, Schadtler-Siwon & Ortiz-Ortiz, 1979), which acted non-specifically on T-cell response, mixed lymphocyte reaction and graft-*versus*-host reaction. However, in most instances, suppressions of cell-mediated immunity has been shown to be

antigen-specifically generated with T cells; they have been observed in contact sensitivity to picryl chloride and to dinitrofluorobenzene (Zembala & Asherson, 1973; Phanuphak *et al.*, 1974; Polak, 1976; Sy *et al.*, 1977) and in cutaneous delayed hypersensitivity to bovine gamma globulin in rats (Ha & Waksman 1973), to collagen in guinea-pigs (Gentner & Adelmann, 1976) and to BCG in mice (Nakamura & Tokunaga, 1980). In our study, live BCG and heat-killed BCG induced suppressor macrophage(-like cells) and suppressor T cells, respectively, and acted antigen non-specifically on DTH suppression. Such antigen non-specific suppressor cells may be characteristic for BCG since it possesses intensive adjuvant activity in its adjuvant portion. We showed that previous i.v. injections with 500 μg of N-acetylmuramyl-L-alanyl-D-isoglutamine, the 'minimum' adjuvant of BCG, in water-in-oil-in-water emulsion could suppress DTH in mice immunized with BCG cell walls (Kato, unpublished). In another study, Hanna *et al.* (1980) found that the non-antigenic adjuvant, $Al(OH)_3$ induced non-specific suppressor cells which inhibited lymphocyte proliferation *in vitro*. Exactly why live BCG induces macrophage-like suppressor cells while, on the other hand, heat-killed BCG induces suppressor T cells is a subject we are continuing to pursue in our laboratory.

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