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Induction of Suppressor T Cells in Delayed-Type Hypersensitivity to *Mycobacterium bovis* BCG in Low-Responder Mice

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The induction of delayed-type hypersensitivity to *Mycobacterium bovis* BCG was specifically inhibited by suppressor T cells in C3H/He, a strain of mice which is a low responder to BCG. The existence of these suppressor cells was confirmed by an adoptive transfer of spleen cells of BCG-injected mice into cyclophosphamide-treated recipients. The suppressor cells appeared in the spleens of the mice 2 to 7 days after intravenous BCG injection. They were sensitive to anti-θ serum and complement and did not adhere to Sephadex G-10. A pretreatment of the mice with cyclophosphamide eliminated the suppression of delayed-type hypersensitivity. These suppressor cells effectively inhibited the induction of delayed-type hypersensitivity to BCG, but showed only weak effect on the expression of it.

There are reports on antigen-specific suppressor cells in delayed-type hypersensitivity (DTH) such as 2,4-dinitro-1-fluorobenzene or picryl chloride in mice (14, 15, 18, 21) to bovine gamma globulin in rats (6) and to collagen in guinea pigs (5). In these cases, the suppressor cells are classified mostly as T cells. On the other hand, there are reports on nonspecific suppressor cell induction by infection with various microorganisms such as *Mycobacterium bovis* BCG, *Corynebacterium, Schistosoma,* or *Trypanosoma* (1, 7, 8, 16). The suppressor cells induced by infection with these microorganisms were macrophages except in the case of *Trypanosoma* infection when they were T cells.

We previously reported that C3H/He mice are low DTH responders to BCG when evaluated by footpad reaction (FPR), and SWM/Ms mice are high responders (11). BCG is well known as an effective adjuvant for enhancing DTH, antibody production, and tumoricidal activity of macrophages (3, 20). At the same time, there are reports of suppressor cell induction by BCG (3, 4, 8). It is interesting to know whether even as strong an adjuvant as BCG could induce suppressor cells under certain conditions. The diversity in the action of BCG must be considered and analyzed on the level of cell-to-cell interaction. However, there has been no report on the induction of suppressor cells specific for BCG itself. In the present paper, we report the evidence for the induction of antigen-specific suppressor cells during the process of BCG injection in C3H/He mice. A possible relationship between suppressor cell induction and strain difference in DTH to BCG is discussed.

MATERIALS AND METHODS

Mice. Inbred C3H/He mice were purchased from Funabashi Animal Farm (Funabashi, Chiba, Japan) or Shizuoka Experimental Animals (Shizuoka, Japan). The animals, four or five in each group, were used at 6 to 12 weeks of age.

BCG. *M. bovis* strain BCG (Japanese substrain) was used. The bacilli were prepared as described elsewhere (11).

Immunization to BCG and evaluation of DTH. Living BCG (10^7 cells in 0.1 ml of saline) were injected subcutaneously into the flanks of mice. Two weeks later, 10 μg of purified protein derivative (PPD; Nihon BCG Laboratory, Tokyo, Japan) in 0.05 ml of saline was injected into a hind footpad of each mouse, and the footpad swelling was measured 24 and 48 h later. Since the footpad swelling with the injection of saline did not persist for 24 h, the FPR was recorded as the difference in thickness before and after the PPD injection, 0.1-mm units. In the experiment to examine the effect of injection routes in immunization, BCG was injected into the mice intravenously (i.v.), intraperitoneally, or into a footpad.

Immunization to SRBC and evaluation of DTH. Sheep erythrocytes (SRBC, purchased from Funabashi Animal Farm) were suspended in saline at a concentration of 2×10^9/ml, and 0.05 ml of the suspension was injected into a hind footpad of each mouse. Four days later, the same amount of SRBC was injected into the counter footpad, and FPR was measured 24 h later.

CY treatment. Cyclophosphamide (CY) was purchased under the trade name Endoxan from Shionogi & Co. Ltd. (Osaka, Japan). CY was injected intraperitoneally into mice at a dose of 200 mg/kg 2 days before immunization.

Suppressor cells. The spleens of C3H/He mice receiving 10^7 BCG i.v. 1 week earlier were used as the suppressor cell source. The spleen cell suspension was

prepared as described elsewhere (11). The cells were washed four times at 120 × *g* for 10 min.

Fractionation of the suppressor cells. The suppressor cells were applied onto a Sephadex G-10 (Phamacia Fine Chemicals, Uppsala, Sweden) column to deplete macrophages (12). Briefly, the cells were suspended in RPMI 1640 medium containing 10% fetal calf serum at a concentration of 2×10^7/ml. A 10-ml amount of the suspension was applied onto a Sephadex G-10 column (50-ml syringe) and incubated at 37°C for 30 min. The nonadherent cells were eluted by warmed medium and used in the transfer experiment.

Adoptive transfer of the suppressor cells. The suppressor cells were transferred i.v. into syngeneic recipients treated by CY previously. To observe the effect of the suppressor cells in the induction phase of immunization, the cells were transferred before immunization. The recipients were injected with BCG and SRBC immediately after the cell transfer. To observe the effect of the suppressor cells in the expression phase of immunization, the cells were transferred 2 weeks after BCG immunization, followed immediately by the footpad test with PPD. In some experiments, the spleen cells of BCG-sensitized mice were transferred locally to normal recipients. The BCG-sensitized mice were prepared by CY treatment and BCG immunization. Spleen cells of these mice (10^7) were mixed with 10^6 BCG and injected into a hind footpad of each of five mice. The counter footpad received these cells without BCG. The footpad thickness was measured 24 h later, and the difference between the two values was recorded as the FPR in the responder cells. The suppressor cells were added to this system to observe their suppressive effect on the responder cells in the local transfer of DTH. This is another experiment to determine the effect of the suppressor cells in the expression phase of immunization.

Antisera and complement. Mouse anti-θ serum was purchased from Litton Bionetics Inc. (Kensington, Md.). Lyophilized guinea pig complement was purchased from Miles Laboratories (Elkhart, Ind.). With the addition of this antiserum and complement, normal lymph node cells of C3H/He mice lost the ability to respond to phytohemagglutinin (Difco, 1 μg/ml) in vitro, but responded well to lipopolysaccharide (Difco, 25 μg/ml).

Statistics. Student's *t* test was used for the statistical analysis of the experimental results.

RESULTS

Enhancement of DTH to BCG in C3H/He mice by pretreatment with CY. CY has been known to enhance cell-mediated immunity by inhibiting the antibody production (9), or by eliminating suppressor cell precursors (17). We have found that the FPR to PPD at 2 weeks of BCG immunization was markedly enhanced when 200 mg of CY per kg was injected intraperitoneally into C3H/He mice 2 days before the immunization. Table 1 shows the results. The difference between the values of FPR in the CY-treated group and the untreated group was statistically significant at 24 and 48 h ($P < 0.01$).

Effect of route of BCG injection on induction of DTH. The route of injection influenced the induction of DTH to BCG in C3H/He mice (Table 2). When live BCG were injected i.v., intraperitoneally, or subcutaneously into C3H/He mice, DTH to BCG was low at 2 weeks of immunization. However, when BCG was injected into a hind footpad, the FPR to PPD in the counter footpad at 2 weeks of immunization was significantly higher than that seen in the mice receiving BCG by other routes.

This observation, taken together with that shown in Table 1, suggested that there might be a suppressor mechanism in BCG immunization in C3H/He mice. The suppression is sensitive to CY treatment and appeared most strongly when BCG was injected i.v. In contrast, it was weak when BCG was administered by the footpad route. Thus, it was presumed that the suppressor mechanism is associated with the spleen. To examine this possibility, a spleen cell-transfer experiment was conducted.

Evidence for the appearance of suppressor cells in the spleen of mice receiving BCG. To determine whether suppressor cells were induced in C3H/He mice receiving BCG i.v., 10^8 spleen cells from these mice were transferred to CY-treated syngeneic recipients, and the recipients were immunized to BCG and SRBC immediately after the cell transfer. DTH to SRBC or to BCG was determined as described

TABLE 1. *FPR to PPD in BCG-immunized C3H/He mice with or without CY treatment*

CY treatment[a]	FPR[b] to PPD	
	24 h	48 h
−	3.50 ± 1.15	2.67 ± 0.54
+	8.80 ± 1.21[c]	6.72 ± 0.38[c]

[a] Intraperitoneal injection of 200 mg of CY per kg 2 days before BCG injection.

[b] Tested at 2 weeks of immunization (0.1 mm ± standard error).

[c] Differs from the control significantly ($P < 0.01$).

TABLE 2. *Effect of route of BCG injection on the induction of DTH in C3H/He mice*

Route of injection[a]	FPR[b] to PPD at 24 h
Subcutaneous	2.63 ± 0.17
Intraperitoneal	3.40 ± 1.26
i.v.	1.20 ± 0.32
Into footpad	6.66 ± 0.61[c]

[a] Injection of 2×10^7 BCG.

[b] Values in 0.1 mm ± standard error.

[c] Differs significantly from any other values ($P < 0.05$).

in Materials and Methods. In some experiments, serum instead of spleen cells was taken from the donor mice and injected i.v. into the recipients. Table 3 shows the results. The spleen cells of C3H/He mice receiving BCG suppressed the footpad swelling in BCG-immunized recipients. This suppressive effect was decreased significantly by treatment of the cells by anti-θ serum and complement before the transfer. The suppression seemed antigen specific, because DTH to SRBC was not suppressed by those suppressor cells. The serum from the suppressor cell donors did not suppress DTH to BCG in the recipients.

The spleen cells of normal C3H/He mice showed a little suppressive effect on DTH to BCG when they were transferred to CY-treated recipients; however, the difference in FPR between this group and the control was not statistically significant ($0.05 < P$).

Kinetics of suppressor cell induction. To determine the time course of suppressor cell induction, the spleen cells were transferred from C3H/He mice receiving BCG i.v. 2, 4, or 7 days earlier into CY-treated recipients. The recipients were immunized to BCG immediately after the cell transfer, and examined for FPR to PPD 2 weeks later. The spleen cells from the donor mice 2 or 4 days after BCG injection showed the suppressive effect (Table 4). It is apparent that suppressor cells are induced in a comparatively short time after BCG injection in C3H/He mice.

Suppressive effect of nonadherent cells to Sephadex G-10. The spleen cells used as the suppressor cells in the previous experiment were fractionated by applying them onto a Sephadex G-10 column. The nonadherent cells, depleted of macrophages (10), were examined for their suppressive effect by transferring them into CY-treated recipients. The recipients were immunized to BCG immediately after the cell transfer. Table 5 shows the result that the macrophage-depleted suppressor cell fraction was as suppressive as the original spleen cells. Almost the same degree of the suppression was caused by the transfer of 2×10^7 nonadherent cells as by the transfer of 10^8 nonfractionated spleen cells. From this result, it is suggested that macrophages are not responsible for the antigen-specific suppression of DTH. The treatment of the nonadherent cells with anti-θ serum and complement eliminated the suppressive effect of this fraction, suggesting that B cells do not have a suppressive effect in this system.

Effect of the suppressor cells on the expression of DTH. Table 6 shows the effect of the suppressor cells on established DTH to BCG. Suspensions of 10^8 suppressor cells were transferred into mice which had been treated by CY and immunized to BCG 2 weeks earlier. The recipients were tested for FPR to PPD immediately after the cell transfer. As shown in Table 6, the established DTH was a little suppressed by these suppressor cells, but the effect was not as strong as seen in the induction phase of DTH.

Table 7 shows the results of the local cell transfer experiment to observe the effect of the

TABLE 4. *Kinetics of suppressor cell induction in C3H/He spleen cells by i.v. injection of BCG*

Days after BCG injection in donors[a]	FPR[b] to PPD	
	24 h	48 h
—[c]	7.30 ± 1.14	7.17 ± 0.95
2	3.95 ± 1.28[d]	2.20 ± 0.48[d]
4	3.87 ± 1.45[d]	2.60 ± 1.36[d]
7	3.50 ± 1.15[d]	2.67 ± 0.54[d]

[a] The transferred spleen cells (10^8/mouse) were prepared from the mice receiving BCG i.v. 2, 4, or 7 days earlier.
[b] Values in 0.1 mm ± standard error.
[c] The recipients received no suppressor cells.
[d] Value differs significantly from the control ($P < 0.01$).

TABLE 3. *Effect of transfer of the cells or the serum from C3H/He receiving BCG i.v. on the induction of DTH to BCG and SRBC*

Group	Transferred agent	FPR[a] to:		
		PPD (24 h)	PPD (48 h)	SRBC (24 h)
1	None	7.52 ± 0.86	7.14 ± 0.95	7.97 ± 1.11
2	Whole spleen cells[b]	3.83 ± 1.45[c]	2.60 ± 1.36[c]	6.36 ± 1.71
3	Whole spleen cells + anti-θ + complement	6.40 ± 1.45[d]	5.35 ± 1.48	8.09 ± 1.57
4	Serum[b]	7.56 ± 2.16[d]	5.93 ± 1.75	ND[e]
5	Normal spleen cells	7.10 ± 1.38[d]	6.26 ± 1.23	7.83 ± 1.01

[a] FPR in recipients, treated with CY 2 days before the cell transfer (0.1 mm ± standard error).
[b] Donor mice received BCG injection i.v. 1 week before sacrifice.
[c] Differs from the control ($P < 0.01$).
[d] No significant difference from the control ($0.05 < P$).
[e] Not done.

TABLE 5. *Suppressive effect of nonadherent cells on the induction of DTH to BCG*

Transferred cells	Dose	FPR[a] to PPD	
		24 h	48 h
None		9.57 ± 2.32	6.25 ± 0.87
Whole spleen cells[b]	1×10^8	5.62 ± 0.99[d]	2.47 ± 0.95[d]
Nonadherent cells[c]	2×10^7	4.50 ± 1.68[d]	3.56 ± 1.39[d]
Nonadherent cells + anti-θ + complement	2×10^7 (equivalent)	8.46 ± 2.06[e]	5.83 ± 3.29[e]

[a] Values in 0.1 mm ± standard error.
[b] Nonfractionated spleen cells from C3H/He mice receiving BCG i.v. 1 week earlier.
[c] Nonadherent fraction of the whole spleen cells applied onto Sephadex G-10 column.
[d] Differs significantly from the control ($P < 0.05$).
[e] No significant difference from the control ($0.05 < P$).

TABLE 6. *Effect of suppressor cells on the expression of DTH to BCG in C3H/He mice*

Transferred cells	FPR[a] to PPD	
	24 h	48 h
None	9.84 ± 1.65	8.26 ± 2.14
Whole spleen cells[b]	7.46 ± 1.50[c]	7.68 ± 2.69

[a] Recipients were treated by CY before BCG immunization. They received the cell transfer on the day of the PPD test. Values in 0.1 mm ± standard error.
[b] Donor mice received BCG injection i.v. 1 week earlier.
[c] The value differs from that of the control ($0.01 < P < 0.05$).

suppressor cells in the expression phase of the immune response. The suppressor cells were mixed with the responder cells from BCG-sensitized C3H/He mice at the same dose, 10^7, and with 10^6 BCG and transferred to hind footpads of normal C3H/He mice. Responder cells with BCG served as the positive control, and suppressor cells or normal spleen cells with BCG constituted the negative control. As shown in Table 7, the suppressor cells cotransferred with the responder cells, and BCG did not suppress the FPR in the recipients significantly ($0.05 < P$), although the value was a little less than that in the positive control. From these results, it is suggested that these suppressor cells have only a weak effect on the expression phase of the DTH. Normal spleen cells cotransferred with the responder cells did not suppress the footpad swelling at the transfer site.

DISCUSSION

In this study, we have shown that BCG-specific suppressor T cells are induced in C3H/He mice, which are low DTH responders to BCG. There are reports on suppressor cell induction in DTH such as in the case of contact sensitivity to 2,4-dinitro-1-fluorobenzene or picryl chloride (14, 15, 18, 21), the skin reaction to bovine gamma globulin in rats (6), or skin reaction to collagen in guinea pigs (5). In most cases, it was confirmed that the effectors in the suppression were T cells and that the suppression was antigen specific (6, 14, 18, 21). In this regard, the character of the suppressor cells in the present study is consistent with that of the suppressor cells mentioned above. However, there are other reports on suppressor cell induction by BCG, *Corynebacterium, Shistosoma,* and *Trypanosoma* infection (1, 7, 8, 16). These suppressor cells are not antigen specific and suppress cell-mediated cytotoxicity or in vitro DNA synthesis induced by mitogens. They have the character of macrophages (1, 7, 8), except those appearing in *Trypanosoma* infection in which the suppressor cells are T cells (16). BCG-induced suppressor T cells were reported recently (4), but these suppressor T cells acted nonspecifically on in vitro T-cell response, graft-versus-host reaction, or mixed lymphocyte reaction. These results, including the ones with BCG injection, seem to differ from ours in the character of the suppressor cells. However, it is possible that there are nonspecific suppressor cells other than T cells induced by BCG injection in our system. Further research is underway.

The routes of administration of BCG influenced strongly the induction of the suppressor cells (Table 2). Injection i.v. was the most effective route for the induction of suppressor cells. The results seem consistent with the report that

TABLE 7. *FPR elicited by the local transfer of spleen cells and BCG*

Group	Transferred cells	BCG	FPR[a] at 24 h
1	None	−	0.06 ± 0.32
		+	1.20 ± 0.16
2	Responder cells[b]	−	0.52 ± 0.22
		+	5.55 ± 0.96
3	Suppressor cells[c]	−	0.97 ± 0.12
		+	1.40 ± 0.72
4	Normal spleen cells	−	1.42 ± 0.53
		+	1.76 ± 0.84
5	Responder cells and suppressor cells	−	1.62 ± 0.68
		+	4.87 ± 0.94[d]
6	Responder cells and normal spleen cells	−	2.11 ± 0.57
		+	5.66 ± 1.03[d]

[a] Values in 0.1 mm ± standard error.
[b] Spleen cells of C3H/He mice receiving CY treatment and BCG immunization.
[c] Spleen cells of C3H/He mice receiving BCG i.v. 1 week before.
[d] No significant difference from the value in group 2 ($0.05 < P$).

the spleen is required for suppressor cell induction (19).

The suppressor cells were examined for their carrying of living BCG into the recipient in the cell transfer experiment. The spleen cells of the donor were washed four times, homogenized, and cultured on Ogawa egg medium to determine the colony-forming units. It was found that about 10^4 colony formants were carried by 10^8 spleen cells. This amount of BCG did not induce DTH in suppressor cells in C3H/He (data not shown).

The suppressor cells were found to appear as early as 2 days after BCG injection (Table 4). It is likely that they appear earlier than the effector cells of DTH and that the target cells of the suppressor cells are the precursors of the effector cells of DTH to BCG, because these suppressor cells affected the expression of DTH only weakly (Table 6 and 7). It might need a large population of the suppressor cells to inhibit the secondary immune response.

Normal spleen cells transferred to CY-treated recipients did not suppress the induction of DTH in the recipents. However, it is possible that the transferred cells involve the precursors of suppressor cells. A slight reduction in FPR to PPD in group 5 in Table 3 may be explained as the result of the transfer of such precursor cells. On the other hand, normal spleen cells had no effect on the expression phase of DTH (Table 7).

We have found that spleen macrophages of C3H/He mice are poor in antigen-presenting ability in the secondary response of BCG immunization (12). There is a report that the low-responder macrophages do not present antigens to helper T cells (2). It would be interesting to know whether or not the defect of antigen-presenting ability of macrophages relates to the suppressor cell induction in C3H/He, a low-responder strain in DTH to BCG.

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