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Mechanisms of BCG Action

I. The Induction of Nonspecific Helper Cells During the Potentiation of Alloimmune Cell-Mediated Cytotoxic Responses

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Summary. *Donor mice were treated IV with BCG and after various time intervals the spleens from these animals were injected into syngeneic recipients which were simultaneously challenged with an allogeneic tumour. The spleen cells from the BCG-treated donors, but not untreated donors, conferred on the recipients an ability to induce a potentiated CMC reaction against the tumour. The transference of BCG-induced potentiating activity could not be explained by the transference of viable BCG organisms, but was mediated by a cell that was anti-Thy.1-sensitive, silica-resistant, plastic-nonadherent, and nylon wool-adherent, and was sensitive in vivo to anti-thymocyte serum but resistant to hydrocortisone. By the use of congenic strains of mice that differed at the Thy.1 allele, it was shown that the cells responsible were not precursors of the cytotoxic lymphocytes but were cells that produced an amplification of the response of the recipient host's precursor cytotoxic T cells.*

Introduction

Bacillus Calmette-Guérin (BCG) has been shown to potentiate several immune reactions [3–5, 11, 12, 20, 27, 30] which are the result of a complex chain of cellular events. Since BCG probably acts by altering the regulation of immune responses at more than one level, the clarification of the immunopotentiating mechanisms of BCG would represent a significant step towards the understanding of such a regulation. Investigations in this laboratory have shown that different BCG regimens produce different levels of potentiation of CMC developed in response to the inoculation of an allogeneic tumour, and that these effects can be correlated to the rejection of syngeneic tumours [11, 12]. Moreover, it was found that BCG was most effective if given in a large IV dose (1,000 μg; $1-3 \times 10^7$ viable units) about 12 days before tumour implant, followed by a low second dose (10 μg) at the time of the implant. The need for the two sequential doses separated by a time interval similar to that required for the full development of delayed-type hypersensitivity to BCG antigens [4] suggested that development of immunity to BCG may play a role in immunopotentiation. In an attempt to clarify the situation, spleen cells from BCG-treated donors were transferred to syngeneic recipients to determine whether the donor spleen cells could influence the CMC response of the recipients to a subsequent challenge by an allogeneic tumour.

Materials and Methods

Animals. Inbred 6- to 8-week-old female DBA/2J mice ($H\text{-}2^d$), AKR mice ($H\text{-}2^k$), and the female hybrids of the first generation of A/J($H\text{-}2^a$) and C57Bl/6J($H\text{-}2^b$), called $B6AF_1$, were obtained from the Jackson Laboratory, Bar Harbor, Maine, USA. A strain of mice congenic with C57Bl/6J, called B6.AKR, was bred in this laboratory. This strain was the result of backcrosses for seven generations to the parental strain C57Bl/6J, starting from a (C57Bl/6J × AKR)F_1 hybrid animal. After each backcross generation, animals carrying the Thy.1.1 antigen of AKR were selected as breeders for the next generation. These seven backcross generations were followed by brother-to-sister matings for four generations, after which all animals of this strain were shown to be homozygous for the $H\text{-}2^b$ haplotype and the *Thy.1.1* allele.

Cell-Mediated Cytotoxicity (CMC) Assay. The assay was performed as previously described [10, 11]. Briefly, mouse spleen cells were mixed with 4×10^4 ^{51}Cr-labelled P815–X2 target cells to give attacking-to-target cell ratios of 100 : 1, 25 : 1, and 10 : 1 in the wells of microtiter plates, and incubated at 37° C for 16 h. The plates were centrifuged at 250 *g* for 10 min and the level of ^{51}Cr in the supernates estimated. The percent corrected lysis was calculated according to the formula (E-C/T-C) × 100, where E = cpm in the supernate of wells containing experimental spleen cells, C = mean cpm in the supernate of wells containing normal spleen cells, and T = mean cpm in the supernate of wells in which 100% target cell lysis was induced by freezing and thawing four times. The results were then expressed in lytic units (LU), where 1 LU was defined as the number of spleen cells required to produce 33% lysis of the target cells, and were calculated by plotting the percent corrected lysis against $\log_{10}$ of the number of lymphocytes in the three doses mentioned above. This value was divided into the total number of lymphocytes per spleen to produce results in their final form, expressed as LU/spleen.

Tumour. Mastocytoma P815-X2, transplantable in DBA/2 mice, was maintained as an ascites tumour and injected IP at a dose of 10^7 cells for the immunisation of the allogeneic $B6AF_1$ mice.

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BCG Vaccine. Freeze-dried vaccine (Connaught Medical Research Laboratories, Toronto, Canada) containing $1-3 \times 10^7$ viable units/mg, was reconstituted in HEPES-buffered Hank's balanced salt solution and made up to the appropriate concentration. The suspension was injected in 0.1 ml volumes.

Selective Removal of Thy.1+ Cells. Spleen cells bearing the Thy.1 alloantigen were destroyed as previously described [11], using either AKR-anti-C_3H serum (anti-Thy.1.2) or C_3H-anti-AKR (anti-Thy.1.1), depending on the genotype of the spleen cell donor.

Selective Removal of Silica-Sensitive Cells. Phagocytic spleen cells sensitive to the action of in vitro crystalline silica were selectively destroyed as previously described [11].

Fractionation of Spleen Cells on Nylon Wool Columns. Spleen cells were fractionated on nylon wool columns using the method of Julius et al. [23].

Viable Counts of BCG in Spleens. After the spleen were removed from BCG-treated donor mice, single-cell suspensions were prepared. Appropriate serial dilutions of the suspension were prepared and plated out on enriched Middlebrook 7H10 agar (Difco Laboratories Ltd, Michigan, USA). The cultures were incubated at 37° C for 28 days, the mycobacterial colonies counted, and the numbers of BCG organisms expressed in colony-forming units (CFU).

Treatment of Donor Mice with Anti-Thymocyte Serum (ATS) and Hydrocortisone. ATS was prepared by immunising New Zealand White rabbits with $B6AF_1$ thymus cells as previously described [2]. The antiserum, injected SC into $B6AF_1$ mice, was titrated to find the maximal dilution that was still immunosuppressive. In the experiments described here, 0.1 ml of a 1/6 dilution of the antiserum was injected SC into the donor mice 48 h before the cell transfer.

A commercial preparation of hydrocortisone (Hydrocortone: Merck, Sharp and Dohme, Quebec, Canada) was injected SC into donor mice at a dose of 150 mg/kg body weight, 48 h before cell transfer.

General Scheme of Experiments. Donor $B6AF_1$ mice received injections of 1,000 μg BCG and 12 days later they were sacrificed and their spleens removed. Single-cell suspensions were prepared and injected IV into syngeneic recipient mice. These recipients were given a simultaneous injection of 10^7 P815 cells IP. The spleens from the recipient mice were examined for CMC activity 15 days after tumour implant.

Results

Transfer of Immunpotentiation with Spleen Cells from BCG-Treated Donors

In the initial experiments, recipient $B6AF_1$ mice challenged IP with 10^7 P815 cells were simultaneously given an IV injection of either 10^8 syngeneic normal spleen cells or 10^8 spleen cells from donors treated with 1 mg BCG IV 12 days earlier. The results (Table 1) demonstrated that the injection of spleen cells from BCG-treated donors significantly increased the response of recipient mice. The injection of normal spleen cells caused a slight increase (statistically nonsignificant) in the response, which was interpreted to be due to the increased presence of T cell precursors in the recipient mice following the injection. It was concluded that the capacity to induce a stronger CMC response was conferred upon the spleen cells by the BCG treatment to the donors. The injection of 10 μg BCG at the same time as the tumour cells did not potentiate any further the response of the mice receiving spleen cells from either normal or BCG-treated donors. Furthermore, only in recipient mice supplemented with cells from BCG-treated donors was it possible to detect increased numbers of cells within the spleens. In this (and in subsequent experiments) the donor spleen cell suspensions were assayed for CMC activity, and in all cases the spleen cells exhibited no intrinsic cytotoxicity (results not given in detail [11]). The donor spleens were also assayed for the presence of BCG CFU as a measure of the number of viable BCG organisms. Under these conditions, 12 days after the injection of 1,000 μg BCG vaccine the donor spleen always contained less than 0.5% of the injected dose. The specificity of the cytotoxic activity in the recipient spleens was also tested, and it was found that the animals exhibited reactivity only against the immunising agent P815, with no or little activity against leukaemia EL_4, melanoma B_{16} and sarcoma I (results not given in detail [11, 13]). Hence the increased CMC reactivity of the recipients was related to the BCG treatment of the donors and was due either to the transfer of a BCG-affected cell population or to the transfer of viable BCG organisms. This latter point was tested directly by

Table 1. Effect of the transfer of spleen cells from BCG-treated and normal donors on the CMC response of tumour-grafted recipient mice

Spleen cell donor[a]	No. of cells per recipient ($\times 10^8$)	Spleen equivalents[b]	BCG dose to recipients (μ)	No. of BCG CFU transferred ($\times 10^4$)	Recipient spleen		
					LU/spleen[c]	Increase in numbers of LU/spleen	No. of cells per recipient spleen ($\times 10^7$)[c]
None (control)	–	–	–	–	94 ± 22	–	22.0 ± 3.0
Normal	1.0	1.0	0	0	159 ± 59	65	20.0 ± 3.2
BCG-treated	1.0	0.33	0	4.4 ± 0.6	427 ± 83*	333	31.5 ± 1.8***
Normal	1.0	1.0	10	0	177 ± 16	83	21.0 ± 2.2
BCG-treated	1.0	0.33	10	4.4 ± 0.6	347 ± 61**	253	31.0 ± 2.0

[a] Spleen cells from normal or BCG-treated (1,000 μg BCG IV 12 days earlier) mice were injected IP into syngeneic $B6AF_1$ recipient mice, which received an IV injection of 10^7 P815 tumour cells. Some of the recipients also received 10 μg BCG IP together with the tumour cells. The controls received only the tumour cells. CMC was performed 15 days after tumour cell implant

[b] Calculated from the formula: Number of cells transferred/mean number of cells per donor spleen

[c] Values are the means ± SEM calculated from individual spleens (3 mice/group). The *P* values were calculated with Student's *t*-test: $P < 0.01$(*); $P < 0.02$(**); $P < 0.05$(***) and compared each experimental group with the control group

injecting artificial mixtures of BCG organisms and normal spleen cells into recipient animals. The results (Table 2) indicated that BCG-related potentiation could not be reproduced simply by administering an IV injection of either BCG alone or a mixture of BCG and normal spleen cells at the time of the tumour implant. This was interpreted as an indication that BCG organisms were unable directly to influence the CMC reaction to a great degree when injected at the time of implant. This confirmed earlier findings [11] which indicated that although BCG administered simultaneously with the tumour produced slight increases in the intensity of the CMC reaction, the level of the response was not always significantly different from the level obtained in the absence of BCG.

In the experiments presented in Table 3, various numbers of spleen cells from BCG-treated donor mice were transferred to tumour-challenged recipients. The results indicated that increases in CMC activity of the recipient mice were dependent on the number of cells transferred. However, since the number of BCG organisms transferred was proportional to the number of cells transferred, the increases in CMC acticity of the recipient could be related to the number of BCG organisms transferred, although the data contained in Table 1 and 2 would argue against this.

Table 2. Effect of BCG injected at the time of the tumour implant

Treatment of recipient[a]	Number of BCG CFU injected[b]	LU/ spleen[c]
None	–	162 ± 18
10^8 normal spleen cells	–	165 ± 32
10^8 spleen cells from BCG-treated donor	5.3×10^4	474 ± 59*
1,000 μg BCG	2.8×10^7	201 ± 8
100 μg BCG	1.4×10^6	192 ± 11
10 μg BCG	1.3×10^5	214 ± 37
1 μg BCG	2.3×10^4	151 ± 43
10^8 normal spleen cells + 1,000 μg BCG	2.8×10^7	226 ± 16
10^8 normal spleen cells + 100 μg BCG	1.4×10^6	168 ± 20
10^8 normal spleen cells + 10 μg BCG	1.3×10^5	179 ± 31
10^8 normal spleen cells + 1 μg BCG	2.3×10^4	178 ± 40

[a] Controls received no treatment, or 10^8 normal or BCG-treated spleen cells. Experimental groups received either BCG alone or BCG mixed with 10^8 normal spleen cells: These were administered IV. All recipients received 10^7 P815 cells IP and the CMC response was measured 15 days later

[b] The figures were obtained by plating out and incubating several aliquots of the reconstituted vaccine on Middlebrook 7H10 agar

[c] The values are the means ± SEM calculated from individual spleens (3 mice/group). The level of LU/spleen was compared with the group receiving no treatment (except the tumour cells) and was calculated by Student's *t*-test: $P < 0.01$(*), all other groups nonsignificant

Effect of Varying the Time of BCG Treatment of Donor Mice

In all the experiments described so far, the donor mice were sensitised to BCG by an IV injection of 1,000 μg BCG vaccine 12 days before the transfer to the recipient mice. This time interval had been found from earlier work to be most effective in potentiating CMC. In the experiments presented in Table 4, the time of sensitisation of the donor animals to BCG was varied from 3 to 15 days. The results indicated that the capacity to transfer the immunopotentiating effects appeared after 6 days of BCG treatment and reached a maximum after 12 days. It was also observed that spleen cells transferred from donor animals 3 days after treatment caused an inhibitory effect on CMC (42% of control). By measuring the number of BCG CFU transferred, it was observed (Table 4) that there was a decrease with time in the numbers of BCG micro-organisms present in the donor spleens. Hence the increases in recipient CMC activity were directly related to the length of time of BCG treatment of the donors, but were indirectly related to the number of BCG organisms.

Demonstration that T Cells are Required for the Transfer of Immunopotentiation

In the experiments presented in Table 5, the spleen cells were treated in various ways prior to their transfer to the recipient mice. The ability of the spleen cells to transfer an increased CMC response was found to be resistant to the action of in vitro crystalline silica, but was abrogated when the cells were treated with anti-Thy.1.2 serum and complement. These results indicated that the cell required for the transfer of BCG-induced immunopotentiation was a T cell. An interesting, but unexplained observation, was that the removal of glass-adherent cells increased the potentiating effects of spleen cells from BCG-treated donors.

Nylon Wool Fractionation of the Transferred Cells

Spleen cells were fractionated on nylon wool columns and the nonadherent and recoverable adherent cells were injected into

Table 3. Effect of different numbers of spleen cells from BCG-treated donors on the CMC response of tumour-grafted recipient mice

Spleen cell donor[a]	No. of cells per recipient ($\times 10^8$)	Spleen equivalents[b]	No. of BCG CFU transferred ($\times 10^4$)	Recipient spleen		
				LU/spleen[c]	Increase in LU/spleen	No. of cells per recipient spleen ($\times 10^7$)
None (control)	–	–	–	192 ± 18	–	26.3 ± 1.5
BCG-treated	0.1	0.03	0.69	285 ± 38	93	27.4 ± 2.8
BCG-treated	0.5	0.15	3.4	371 ± 34	179*	30.0 ± 3.7
BCG-treated	1.0	0.31	7.1	484 ± 81	292**	33.1 ± 3.1

[a] Various number of spleen cells from BCG-treated (1,000 μg) $B6AF_1$ donor mice were injected IV into the recipients, which simultaneously received 10^7 P815 cells IP. The controls received only P815. The CMC was measured 15 days after implant

[b] Calculated from the formula: Number of cells transferred/number of cells per donor spleen

[c] Values are the means ± SEM calculated from individual spleens (3 mice/group). The significance was calculated by Student's *t*-test, comparing the experimental groups with the control; $P < 0.01$(*); $P < 0.005$(**); all other groups not significant

Table 4. Effect of varying the time of injection of BCG to donor mice

Spleen cell donor[a]	Days after BCG injection	No. of cells per recipient ($\times 10^8$)	Spleen equivalents[b]	No. of BCG CFU transferred ($\times 10^4$)	Recipient spleen			
					LU/spleen[c]	P[d]	No. of cells per recipient spleen ($\times 10^7$)[c]	P[d]
None (control)	–	–	–	–	280 ± 60	–	21.7 ± 1.5	–
Normal	–	1.0	0.96	–	312 ± 32	NS	23.4 ± 2.4	NS
BCG-treated	15	1.0	0.36	6.8	602 ± 43	< 0.005	29.3 ± 1.4	< 0.01
BCG-treated	12	1.0	0.28	5.8	660 ± 48	< 0.005	30.1 ± 2.0	< 0.01
BCG-treated	9	1.0	0.51		425 ± 48	< 0.05	25.3 ± 0.8	NS
BCG-treated	6	1.0	0.54	70.0	480 ± 71	< 0.05	28.3 ± 2.7	NS
BCG-treated	3	1.0	0.53	111.0	120 ± 40	< 0.05	22.9 ± 3.4	NS

[a] Spleen cells from BCG-treated mice (1,000 μg) were injected IV recipients at the times after BCG injection of the donors as indicated in the second column. Comtrols received either no spleen cells or cells from normal animals. All recipients received 10^7 P815 cells IP and the CMC assay was performed 15 days after implant

[b] Calculated from the formula: Number of cells transferred/number of cells per donor spleen

[c] Values are the means ± SEM calculated from individual spleens (3 mice/group)

[d] P values were calculated by Student's t-test by comparing each experimental group with the control group. NS indicates difference was not significant

Table 5. Nature of the cell transferring the BCG potentiating effect

Spleen cell donor[a]	Treatment of spleen cells	Recipient spleen		
		LU/spleen[b]	Increase in LU/spleen	P[c]
None (control)	–	130 ± 26	–	–
BCG-treated	Normal mouse serum + complement	284 ± 40	154	< 0.01
BCG-treated	Anti-Thy.1.2 serum + complement	144 ± 20	12	NS
BCG-treated	Silica	276 ± 78	146	< 0.01
BCG-treated	Glass-adherent cells removed	380 ± 48	250	< 0.005

[a] Spleen cells were obtained from mice treated with 1,000 μg BCG IV 12 days earlier. The cells were divided into 10^8-cell aliquots and were incubated (i) with normal mouse serum and complement; (ii) with AKR-anti-C_3H serum and complement; (iii) with 5 mg crystalline silica for 4 h; or (iv) for 1 h in glass petri dishes for the removal of adherent cells. The cells were washed three times and injected into the recipients which also received 10^7 P815 cells IP. The CMC assay was performed 15 days after the implant

[b] Values are the means ± SEM calculated from individual recipient spleens (3 mice/group)

[c] Values were calculated by Student's t-test by comparing each experimental group with the control group. NS indicates that the difference was not significant

each recipient mouse in doses corresponding to the yield from one-third of a donor spleen. Another group of mice received the two column fractions remixed together. The controls received either no spleen cells or the equivalent of one-third of a donor spleen from the original unfractionated suspension.

The unfractionated spleen cell population and the adherent cell fraction induced significant augmentation of the response of the recipients (148 and 122 LU/spleen, respectively), while the nonadherent cell fraction resulted in only a small increase in the response (31 LU/spleen) (Table 6). When the adherent and nonadherent fractions were recombined prior to their injection into the donor mice, the level of activation produced was equivalent to that produced with the adherent cells alone (134 LU/spleen). Since equivalent amounts of unfractionated cells and of nylon wool column-fractionated cells had been injected into the recipient animals, it was concluded that the activity of the unfractionated cells was accounted for solely by the recoverable nylon wool-adherent cells. Moreover, the number of cells per recipient spleen increased only in those animals that received either the unfractionated or the adherent donor spleen fractions.

Effect of ATS and Hydrocortisone

Mice that had been treated with 1,000 μg BCG were injected with either ATS or hydrocortisone 2 days before the transfer of one-half spleen equivalent into recipient mice. Furthermore, once removed from the donors, the cell populations were fractionated on nylon wool columns. Each fraction was injected into the recipients in a number equivalent to a half spleen. The results, presented in Table 7, indicated that spleen cells from donors treated only with BCG induced a significant increase in the response of the recipients (323 LU/spleen). A similarly increased CMC response was obtained in the recipients of spleen cells from mice treated with BCG and hydrocortisone (295 LU/spleen), while the treatment with ATS severely reduced the increase (54 LU/spleen) and abolished the potentiating effects of BCG. If the cell populations were fractionated on the nylon wool, it was observed that the potentiating properties of hydrocortisone-resistant cells resided in the nylon wool-adherent fraction, producing an increase of 347 LU/spleen compared with 39 LU/spleen observed with the nonadherent fraction.

Table 6. Effect of nylon wool column fractionation on transferred cells

Spleen cell fraction transferred[a]	No. of cells per recipient ($\times 10^8$)	Recipient spleen				
		LU/spleen[b]	Increase in LU/spleen	P[c]	No. of cells per recipient spleen ($\times 10^7$)[b]	P[c]
None (control)	–	122 ± 24	–	–	22.8 ± 0.1	–
Whole, unfractionated	0.71	270 ± 40	148	< 0.02	29.5 ± 2.5	< 0.05
Adherent	0.10	244 ± 2	122	< 0.01	28.5 ± 1.2	< 0.01
Nonadherent	0.26	153 ± 37	31	NS	22.8 ± 2.0	NS
Adherent + nonadherent	0.10 + 0.26	256 ± 20	134	< 0.02	30.6 ± 1.3	< 0.005

[a] Spleen cells from BCG-treated B6AF$_1$ donors (1,000 μg) were separated into adherent and nonadherent fractions by passing them through nylon wool columns in numbers equivalent to one donor spleen per column. Whole, unfractionated cells, the separated fractions, and the reconstituted population (adherent + nonadherent) were injected into recipients in numbers equivalent to one-third of a donor spleen. The recipients then received 10^7 P815 cells IP and the CNC assay was performed 15 days later

[b] Values are the means ± SEM calculated from individual spleens (3 mice/group)

[c] P values were calculated by Student's t-test by comparing each experimental group with the control group. NS indicates that the difference was not significant

Table 7. Effect of ATS and hydrocortisone treatment of the spleen cell donors

Spleen cells transferred to recipient[a]	Treatment of donor	No. of cells per recipient ($\times 10^8$)	Spleen equivalents[b]	Recipient spleen		
				LU/spleen[c]	Increase in LU/spleen	P[d]
None (control)	–	–	–	98 ± 11	–	–
Unfractionated	None	1.0	0.32	421 ± 43	323	< 0.01
Unfractionated	ATS	0.91	0.5	152 ± 73	54	NS
Unfractionated	Hydrocortisone	0.83	0.5	393 ± 4	295	< 0.01
Adherent	ATS	0.37	0.5	134 ± 30	36	NS
Adherent	Hydrocortisone	0.29	0.5	445 ± 80	347	< 0.01
Nonadherent	ATS	0.18	0.5	120 ± 9	22	NS
Nonadherent	Hydrocortisone	0.31	0.5	137 ± 21	39	NS

[a] Spleen cells were obtained from mice treated with BCG (1,000 μg IV 12 days before sacrifice) followed 10 days later by 0.1 ml ATS SC (diluted 1/6), 3 mg hydrocortisone IP, or no other treatment (as control). Spleen cells from the donors were either used unfractionated or separated into adherent and nonadherent nylon wool column fractions. These were injected into recipients in numbers equivalent to one-half of a spleen (unless otherwise stated). The recipient were given 10^7 P815 cells IP and the CMC assay was performed 15 days later

[b] Calculated from the formula: Number of cells transferred/number of cells per donor spleen

[c] Values are the means ± SEM calculated from individual spleens (3 mice/group)

[d] P values were calculated by Student's t-test comparing each experimental group with the control group. NS indicates that the difference was not significant

Roles of Donor and Recipient Cells in the Potentiated CMC Response

To establish whether the cells capable of transferring the immunopotentiating effects of BCG acted as precursors of the cytotoxic T lymphocytes responsible for CMC, congenic strains of mice were used. The two strains C57Bl/6J and B6.AKR are congenic and share the same *H-2*^{b} haplotype. They differ in the Thy.1 alloantigen, the allele of C57Bl/6J being *Thy.1.2,* while that of B6.AKR is *Thy.1.1.* Therefore, the T cells of the two strains can be distinguished by their sensitivity to C_3H-anti-AKR or to AKR-anti-C_3H sera. In two different experiments, animals of C57Bl/6J or B6.AKR strains were treated with 1,000 μg BCG and 12 days later their spleen cells were transferred into recipient animals of the congenic partner strain, which were simultaneously challenged with the tumour. Fifteen days later the recipients' spleen cells were treated with either normal mouse serum or anti-Thy.1.1 or anti-Thy.1.2 serum and the level of CMC measured. The results (Table 8) demonstrated that transfer of BCG immunopotentiation occurred between the two congenic strains and was of the same order of magnitude as that demonstrated in the previous experiments between syngeneic mice of the C57Bl/6J strain. The cytotoxic activity of cells from C57Bl/6J recipients was markedly reduced with anti-Thy.1.2 serum but not with anti-Thy.1.1 serum, while in the reciprocal experiment the cytotoxic activity of cells from B6.AKR recipients was reduced only by treatment with the anti-Thy.1.1 serum and complement. Hence in animals which had received the injection of spleen cells from BCG-treated congenic donors, the results demonstrated that the majority of the cytotoxic T lymphocytes was of the recipient genotype. This indicated that the spleen cells from BCG-treated congenic donors had induced an amplification of the recipients' CMC reaction by supplying cells other than precursors of the cytotoxic lymphocytes. While the B6.AKR strain, having been backcrossed for only seven generations, may not be fully congenic with C57Bl/6, this possibility does not weaken such a conclusion.

Table 8. Contribution to the BCG-stimulated recipient CMC response of donor and recipient effector T-cells

Donor animals[a]	Recipient animals	No. of cells per recipient ($\times 10^7$)	LU/recipient spleen following treatment with[b]		
			No treatment	Anti-Thy.1.2 serum + complement	Anti-Thy.1.1 serum + complement
None (control)	C57Bl/6J *(Thy.1.2)*	–	116 ± 8	13 ± 13	163 ± 8
B6.AKR *(Thy.1.1)*	C57Bl/6J *(Thy.1.2)*	2.5	311 ± 71	43 ± 12	316 ± 46
None (control)	B6.AKR *(Thy.1.1)*	–	153 ± 16	139 ± 23	27 ± 14
C57Bl/6J *(Thy.1.2)*	B6.AKR *(Thy.1.1)*	2.5	403 ± 42	381 ± 4	43 ± 28

[a] Mice of C57Bl/6J or B6.AKR strain were treated with BCG (1,000 μg) IV 12 days before sacrifice. The spleen cells were fractionated on nylon wool columns and the adherent fraction was into the animals of the corresponding congenic strain, which also received 10^7 P815 cells IP. The recipients' spleens, prior to the CMC assay, were treated with nothing, AKR-anti-C_3H serum + complement, or C_3H-anti-AKR serum + complement

[b] Values are the means ± SEM calculated from individual spleens (3 mice/group)

Discussion

The results reported here demonstrate the transfer of immunopotentiating activity with spleen cells from BCG-treated donors. For this phenomenon to be ascribed to a particular cell population, the possibility that the observed increases in the CMC activity of the recipient were due to the transference of BCG organisms has to be eliminated. This latter effect can be excluded, on the basis of several pieces of evidence:

(i) There were very low counts of viable BCG organisms in the donor spleen suspensions at the time of transfer (Tables 1, 3, and 4).

(ii) To produce a significant increase in CMC, BCG must be injected several days before tumour inoculation. When BCG was injected directly into recipients at the same time as the tumour, with or without normal spleen cells, no potentiation was observed (Tables 1 and 2).

(iii) An interval of least 6 days is required between the injection of BCG and the transfer of the spleen cells for the transfer of immunopotentiation. At this time the number of viable BCG organisms has already decreased in comparison to that found 1–3 days after BCG injection. Although BCG injected into mice is able to multiply and divide, Lefford [25] has demonstrated that large initial inocula, as used in the present report, do not tend to increase in number. Hence the best time to transfer the spleen cells, which was 12 days after the injection, does not coincide with an increase in BCG numbers within the spleen (Table 4). While intact BCG may be excluded as the agent that produces potentiation in mice, the involvement of a product derived from BCG after some hypothetical processing step in the primary host or simply as a breakdown product cannot be excluded with certainty.

(iv) The transfer of CMC potentiation requires the active participation of a splenic T cell (Table 5). The possibility that the BCG (whole organisms of subcellular fractions) is associated with the T cells due to natural affinity, and hence eliminated with them, is doubtful because although the cells are extensively washed after anti-Thy.1 treatment, the dead cells probably remain in the injection mixture. Further, the natural affinity ascribed to bacteria and their products is not limited to any particular cell type, but can be demonstrated against a wide variety of mammalian cells (14331). On this basis it would therefore be difficult to explain the differential effects of time (Table 4), nylon wool fractionation (Table 6), and selective immunosuppressants (Table 7). Further, since the T cells have to be alive this would argue against the possibility that these cells act only as passive carriers of BCG.

The anti-Thy.1 sensitivity, the silica resistance, and the lack of adherence to glass surfaces support the identification of the cells responsible for the transfer of CMC potentiation as T cells. Crystalline silica is known to be toxic to macrophages [1] and has previoulsy been shown to be effective in abolishing macrophage functions under the same conditions used in these experiments [19]. However, the identification of the responsible cell as a T cell could be complicated, since some natural killer cells have been shown to express low levels of the Thy.1 antigen on their surface [21, 26]. Further, the cell responsible for the transfer of CMC potentiation was shown to be sensitive to the in vivo administration of ATS and resistant to hydrocortisone. ATS, given at low doses as in the present experiments, affects primarily recirculating T cells, a subset of which includes the precursors of the cytotoxic T cells [2] and helper cells [28]. Hydrocortisone, at the current dose levels, is toxic to some T cells, including a cell possessing amplifier activity in the CMC reaction [2], while it does not affect other more mature T cells, including the precursors of the cytotoxic lymphocytes [2, 7] and educated helper cells [7]. Some caution should be exercised in interpreting these in vivo results, since the BCG treatment may have affected the migratory properties of some cells and their sensitivity to hydrocortisone [7]. Finally, the cell responsible for the transfer was nylon wool-adherent. Nylon wool-adherent T cells have been ascribed several functions, including suppressor activity [18, 22], rosette-forming activity [9], and antigen-induced proliferation in delayed hypersensitivity [29], and have been shown to exhibit different properties from nonadherent T cells [15, 16, 24, 29].

The increased CMC reaction of the recipients of spleen cells from BCG-treated donors may have been expected to be the result of the transfer of large numbers of precursors of the effector cells. This was shown not to be the case in experiments in which the injection of BCG-treated congenic spleen cells differing in the *Thy.1* allele induced a significantly increased CMC reaction, which was almost entirely due to effector cells of the recipient host Thy.1 serotype. It is likely that when whole spleen cell populations are transferred the donor cells may conribute significant numbers of precursors to the host immune reaction and could account for the slight increases observed in CMC reactivity following the transference of normal spleen cells (Tables 1 and 4). However, in the experiments reported in Table 8, in which nylon wool-adherent cells were transferred, it is probable that precursor cells was eliminated with the nonadherent fraction. The CMC

reaction has been shown to involve, both in vivo and in vitro, in addition to the precursors of the cytotoxic cells, the participation of cells with specific helper function [6, 34] which are probably distinct from other cells, are also capable of leading to an increased response, and which have been described as amplifier [2, 17] or initiator [8] cells. The characteristics of ATS sensitivity and hydrocortisone resistance make the cells responsible for the transfer of CMC potentiation more similar to the specific helper cell, while their adherence to nylon wool is not a property usually ascribed to helper cells. However, as discussed above, these characteristics may not have the same significance in BCG-treated animals as in normal ones, since BCG is known to affect the distribution of lymphocytes throughout the body compartments [35]. The cells responsible for the transfer of immunopotentiation may be activated by specific or nonspecific mechanisms, although they appear to exert a nonspecific effect in the recipient mice.

While immunopotentiation was obtained with the transfer of BCG-activated spleen cells, the recipients of such cells did not present any further amplification of the response upon exposure to BCG (Table 1), although it has previously been shown [11] that two injections of BCG resulted in a synergistic effect on the potentiation of CMC responses. For this effect to occur a time interval of several days between the two injections was necessary. Since the transfer of spleen cells from mice treated with BCG induced an amplification of the response, it was expected that a direct exposure to BCG of the recipients following transfer might have increased the response. The reasons why this effect was not observed were not established. Further the levels of potentiation in the recipient mice following transfer were not as great as those expected if BCG had been injected into the recipients directly 12 days before tumor implant [11, 13]. Finally, the level of the CMC response induced in recipient mice treated with either the tumour alone or the tumour and spleen cells from BCG-treated donors was found to vary considerably, ranging from 94 to 280 LU/spleen in the untreated tumour-challenged mice and from 270 to 660 LU/spleen in the potentiated mice. This variation was observed in earlier studies [13] and was found to reflect the differential stimulation of cytotoxic T cell subpopulations. These latter observations stress the complexity of the effects induced by BCG and suggest that more than one mechanism of amplification of the immune response may be stimulated by this immunopotentiator. It is possible that only one of these amplifier mechanisms was transferred in the experiments reported here.

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