Clin. exp. Immunol. (1979) 35, 36–44.

Effects of *Corynebacterium parvum* and BCG therapy on immune parameters in patients with disseminated melanoma. A sequential study over 28 days

I. CHANGES IN BLOOD COUNTS, SERUM IMMUNOGLOBULINS AND LYMPHOID CELL POPULATIONS

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(*Received* 28 *July* 1978)

SUMMARY

The effects of a single immunization of melanoma patients with BCG or *C. parvum* on blood counts, serum immunoglobulin levels and lymphoid subpopulations were followed by multiple assays over 28 days. *C. parvum* produced a decrease in the white cell count, lymphocyte count and lymphoid T and sIg^+ cell numbers, which recovered within 1 week; BCG did not produce such a marked depression. Both agents were associated with increases in T cell numbers and lymphocyte PHA blastogenesis after the first week; these declined to pre-immunization values by 3–4 weeks. The sIg-bearing cell subpopulation also increased after BCG. Different methods of expressing the results were compared and the difficulties of immunological monitoring are discussed.

INTRODUCTION

The rational use of immunomodulators in human malignancy requires documentation of the effects of such agents on immune reactivity in cancer patients. This is important, as increasing numbers of tumour immunotherapy studies are now being undertaken.

The purpose of this and the following report is to describe the effects of *C. parvum* and BCG on various immune parameters, e.g. lymphoid cell subpopulations and 'killer-cell' cytotoxicity, over a 28 day time course. The difficulties of immunological monitoring and the scheduling of immunotherapy with *C. parvum* and BCG are discussed.

MATERIALS AND METHODS

Subjects. Twenty-four consecutive patients with disseminated melanoma were studied, after informed consent had been obtained. No patient had received previous chemotherapy or radiotherapy. Any general anaesthetic had been given at least 14 weeks prior to the study. The median age was 43 years, range 22–75 years. Alternate patients were given either BCG or *C. parvum*; clinical details are shown in Table 1. All patients had a Karnofsky performance score >60.

Immunization. BCG vaccine, percutaneous Glaxo was reconstituted with 0·3 ml sterile water and administered by 'multiple puncture gun'. Five applications of vaccine (100 needle punctures, 2·0 mm depth) were given to each limb.

C. parvum (Burroughs-Wellcome CN6134) 2·0 mg/m² body surface area was given i.v. in 300 ccs of saline over 3 hr. No anti-pyretics nor steroids were given during the treatment.

Thirty-six healthy subjects (the majority blood donors) were employed as normal controls. The median age was 42·5 years, range 22–71 years. Venepuncture was performed immediately before immunization and then at days 2, 4, 7, 10, 14, 21 and

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0099-9104/79/0010-0036$02.00

TABLE 1. Clinical data for patient groups

C. parvum group									BCG group								
					Survival (weeks)		Skin test*							Survival (weeks)		Skin test*	
Patient	Age	Sex	Site	Metastases	1	2	Pre-CP	Post 28 days	Patient	Age	Sex	Site	Metastases	1	2	Pre-BCG	Post 28 days
JW	41	F	Thigh	D,ST,N	216	31	0	1	JB	51	F	Calf	D,N	26	9	1	0
ET	28	M	Finger	D,ST,N	260	39	0	1	EA	55	F	Abdomen	ST,N,P	204	26	1	2
EW	44	M	Arm	D,N	208	76(a)	1	2	ET	56	M	Foot	ST,N	120	74(a)	2	1
ER	58	F	Arm	D,ST,N	220	84(a)	0	2	RB	47	M	Neck	D,ST,N	244	43	0	0
JM	44	F	Shoulder	ST,N,	56	54	1	0	MR	31	F	Calf	D,ST,N	124	68(a)	1	2
NL	33	F	Trunk	D,N,P	52	32	1	0	WK	37	M	Arm	ST,N,H	14	10	2	1
VH	49	F	Scalp	D,ST,N,P	50	32	2	1	MW	75	F	Foot	D,ST,N	52	22	1	0
AB	39	F	Shoulder	ST,N,O	92	88(a)	0	3	ET	34	M	Buttock	ST,N,H,O	30	18	0	1
CM	41	F	Leg	ST,N,H	88	7	2	1	LB	42	F	Scalp	D,ST,N	20	14	1	0
RG	22	M	Foot	D,ST,N,P	96	10	1	0	BS	40	M	Thigh	D,ST,N,P	51	47	0	1
ES	55	F	Back	ST,N	30	22	2	1	EC	53	F	Shoulder	D,ST,N	43	30	2	1
FT	48	F	Leg	D,ST,N	128	120(a)	1	2	AA	35	M	Abdomen	ST,N,P	136	116(a)	0	1

* Skin test — number positive using three antigens.
D = Skin, ST = soft tissue, N = node, P = lung, O = bone and H = liver.

Survival 1 = interval from initial diagnosis to death, or if alive (a) to 30.10.77.
Survival 2 = interval from immunization to death, or if alive (a) to 30.10.77.

28 following immunization. An extra sample at 3 hr was taken at the end of the *C. parvum* infusion. Total white cell, lymphocyte and monocyte counts were routinely estimated for each sample. Serum immunoglobulins IgA, IgM and IgG were measured (Mancini radial immunodiffusion) at the beginning and end of the study period.

Isolation of lymphocytes. Heparinized blood was incubated with finely divided iron and lymphoid cells separated on a Ficoll–Triosil gradient (Thatcher *et al.*, 1977). Differential cell counts on stained smears gave 94% or greater lymphocyte purity.

E-rosettes. The method has been described previously (Thatcher *et al.*, 1977). Briefly, washed SRBC in foetal calf serum (2×10^8 ml) were added to the lymphocyte suspension (40 : 1 cell ratio), incubated at 4°C for 18 hr and the percentage of rosetting cells counted. All preparations were tested in triplicate.

Surface immunoglobulin determination. Lymphocytes were washed three times at room temperature and incubated at 4°C for 30 min with a fluorescent polyvalent, sheep anti-human Ig serum (Wellcome-Reagents Ltd.). After incubation and washing, the cells were examined under a Vickers Photoplan Fluorescence Microscope. The percentage of cells exhibiting surface fluorescence (multiple point staining) was determined.

The null cell percentage was obtained from: 100 − sum (E-rosette cell % + sIg staining cell %). Absolute cell counts were obtained by multiplication with the peripheral lymphocyte count.

PHA stimulation. Lymphocytes were cultured in Cooke microplates at 3×10^5 cells per well with 0·2 ml $NaHCO_3$ buffered minimum essential media (Gibco) supplemented with 10% inactivated FCS, either with or without PHA (Wellcome-Reagents Ltd.) added to give 0·5, 1·0, 2·0 and 6·0 μg/ml. The method, using a 3 day incubation, 6 hr ^{3}H-thymidine pulse and a multiple culture harvester, has been described previously (Potter & Moore, 1977).

The spontaneous ^{3}H-thymidine uptake with media alone and the maximum incorporation with PHA were expressed as mean counts per minute, ct/min and used to derive the 'stimulation index' (SI):

$$\frac{\text{maximum ct/min} - \text{spontaneous ct/min}}{\text{spontaneous ct/min}}.$$

The 'mitogenic capacity' was calculated as Log_{10} (lymphocyte count/ml × [maximum PHA response − spontaneous uptake in ct/min]) (Campbell *et al.*, 1973).

The optimal adjusted PHA response (OAR) is the greatest adjusted PHA response of the four PHA concentrations. The adjusted PHA response is the ^{3}H-thymidine uptake in ct/min multiplied by the negative log of the PHA concentration (mg) in that culture (Gross & Eddie-Quartey, 1976). Calculation of results and statistical analysis was by computer.

RESULTS

Pre-immunization patients' immunological profile compared with a normal control group

These differences were analysed for 'overall' statistical significance among the three groups (BCG, *C. parvum* and controls) by a Kruskall–Wallis one-way analysis of variance and, if significant ($P < 0{\cdot}05$), then the differences between pairs (e.g. BCG *vs* normal control) were analysed by Mann–Whitney U two-tailed tests at a reduced significance level ($P < 0{\cdot}0171$). There were no significance differences for any pre-immunization test value between the *C. parvum* and BCG groups. The median values and ranges (at 0 ↓ time) for the melanoma patients are given in Tables 2, 3 and 4.

There were statistically significant differences for the percentage of E-rosettes between the normal controls (median 72·0, range 50·0–87·0) and the melanoma patients in the BCG and *C. parvum* groups. The E-rosette absolute count exhibited a similar pattern of statistically significant differences (control group, median 140·4, range 72·4–256·7).

The sIg-bearing cells in the melanoma patients were not statistically significantly reduced when compared with the control group; the null cells in the melanoma patients had correspondingly higher median values than in the controls, but again these differences were not statistically significant.

The PHA blastogenesis response was analysed for the five PHA concentrations 0, 0·5, 1·0, 2·0 and 6·0 μg/ml. The responses at the 1·0, 2·0 and 6·0 μg levels were significantly lower in the patient groups compared with control values. The maximum uptake usually occurred with 1·0 μg/ml PHA, the control median value was 78,425 ct/min, range 23,688–203,747. The stimulation index (control median 68·8, range 23·3–331·7), mitogenic capacity (control median 8·201, range 7·608–8·639) and optimal adjusted response (control median 5·413, range 4·893–5·923) were all statistically significantly lower in the patient groups. The blood counts and serum immunoglobulin levels in the melanoma patients were not statistically significantly different from those of the controls, although the median lymphocyte counts in the patient groups were lower than in the control group.

TABLE 2. Peripheral blood counts (medians, ranges and statistical differences)

Day	0↓	(3 hr)	2	4	7	10	14	21	28
Total white cell count ($\times 10^9$/l)									
BCG (n.s.)	5·95	—	5·85	6·50	6·05	6·45	6·00	6·00	5·80
	2·2–9·3	—	2·5–10·7	2·9–8·9	2·3–10·0	2·4–9·9	2·2–11·0	2·3–10·1	2·0–8·5
C. parvum†	7·05	6·15	4·55§	6·30	8·95	7·35	7·75	6·60	6·90
	4·7–10·3	4·1–9·2	3·7–7·9	3·6–7·8	5·5–12·7	4·8–12·6	4·1–10·3	3·1–8·9	4·4–9·4
Lymphocyte count ($\times 10^9$/l)									
BCG (n.s.)	1·46	—	1·21	1·52	1·66	1·97	1·40	1·48	1·57
	0·62–2·32	—	0·58–2·68	1·13–2·18	0·64–2·88	0·70–3·12	0·34–2·70	0·41–3·23	0·62–2·64
C. parvum‡	1·74	0·69§	0·56	1·39	2·13	2·11	1·89	2·30	2·25
	0·86–3·06	0·25–2·07	0·35–2·00	1·09–2·17	1·40–5·51	1·24–4·57	1·58–2·96	1·28–3·03	0·75–3·95
Monocyte count ($\times 10^9$/l)									
BCG (n.s.)	0·27	—	0·25	0·30	0·29	0·20	0·24	0·20	0·32
	0·08–0·62	—	0·12–0·75	0·07–0·51	0·04–0·70	0·13–0·85	0·06–0·62	0·05–0·62	0·08–0·65
C. parvum (n.s.)	0·43	0·28	0·21	0·35	0·37	0·46	0·52	0·43	0·32
	0·06–0·62	0·07–0·53	0·07–0·52	0·13–1·15	0·12–0·95	0·06–1·06	0·16–1·03	0·07–1·09	0·24–0·74

↓ = Immunization.
n.s. = not statistically significant.
Friedman's two-way analysis of variance * $P = 0{\cdot}05$–$0{\cdot}001$, † $P = 0{\cdot}0009$–$0{\cdot}0001$, ‡ $P = 0{\cdot}00009$–$0{\cdot}00001$.
Wilcoxon matched pairs signed rank's test compared change from day 0 for each post-immunization occasion at reduced level of significance; $P < 0{\cdot}01$.
§ Significant change at indicated occasion.

TABLE 3. Subpopulation enumeration (medians, ranges and statistical differences)

Day	0↓	(3 hr)	2	4	7	10	14	21	28
E-rosette cell (%)									
BCG*	59·05	—	65·15	69·1	67·25	70·6§	65·75	70·75§	66·5§
	52·0–69·5	—	52·7–78·6	57·1–75·4	53·6–78·0	53·1–81·3	54·2–80·0	56·0–81·2	53·1–79·8
C. parvum (n.s.)	60·7	58·55	61·85	65·85	65·50	67·0	65·3	64·3	65·05
	44·9–71·9	40·9–75·5	44·3–75·0	41·9–81·0	50·0–79·9	51·7–82·0	52·4–82·5	20·0–77·6	47·0–79·5
sIg cell (%)									
BCG*	21·3	—	23·4	23·6	24·75	24·3	26·65§	23·65	22·15
	7·8–35·9	—	9·9–32·7	12·3–31·4	15·4–33·8	12·2–36·2	13·7–41·0	15·4–38·2	11·8–40·5
C. parvum (n.s.)	28·0	22·0	26·1	29·2	24·3	27·15	24·95	26·2	26·75
	26·0–34·0	6·0–29·0	18·0–33·1	18·0–32·0	21·5–35·0	21·0–34·0	17·0–30·4	20·9–38·0	21·0–37·0
Null cell (%)									
BCG†	19·75	—	10·40	11·30	7·83§	2·25§	7·92§	5·55§	9·85§
	10·7–24·1	—	1·17–37·4	1·13–30·2	−1·77–22·1	−0·9–34·7	−2·2–23·0	−6·07–10·77	−0·57–23·5
C. parvum (n.s.)	10·78	20·90	10·22	9·03	6·83	4·73	7·33	9·60	9·05
	1·7–25·4	0·6–38·5	2·5–29·5	−3·5–29·2	−3·8–22·0	−1·7–18·8	−1·8–22·7	−2·9–27·9	0·3–25·0
E-rosette cells (10^7/l)									
BCG (n.s.)	92·0	—	79·0	108·9	125·0	139·6	108·2	107·7	117·1
	40·7–140·1	—	42·3–167·0	84·3–146·3	34·3–191·8	54·7–229·9	23·2–193·6	31·3–244·5	44·5–179·7
C. parvum‡	85·5	48·0§	33·7§	79·8	138·9§	135·5§	118·9§	143·3	154·7
	68·3–188·5	24·8–136·8	21·1–102·8	51·1–169·3	82·5–440·3	89·3–350·1	83·3–216·2	43·0–225·0	60·9–262·4

Table 3 (*contd.*)

Day	0↓	(3 hr)	2	4	7	10	14	21	28
sIg cells (10^7/l)									
BCG (n.s.)	37·4	—	32·0	36·7	38·3	43·0	43·7	33·8	38·8
	6·5–52·4	—	9·0–55·2	15·9–48·7	15·6–90·7	13·9–98·8	7·0–65·5	6·3–83·3	10·4–106·9
C. parvum‡	51·0	12·8§	13·8§	39·3	62·1	53·8	52·5	59·1	52·9
	28·5–79·6	8·2–32·8	8·4–36·5	19·6–48·9	36·5–135·5	26·0–107·9	32·0–75·1	32·5–115·1	23·1–146·2
Null cell (10^7/l)									
BCG†	27·5	—	12·0	15·4	12·8§	4·9	8·0§	6·3§	13·8§
	9·5–45·9	—	1·4–82·7	1·7–53·5	−4·1–33·9	−2·8–97·2	−1·7–28·1	−9·0–17·1	−1·3–22·4
C. parvum (n.s.)	14·5	11·7	5·1	14·5	17·3	9·6	14·0	20·1	25·5
	1·8–45·7	0·5–63·3	1·2–43·0	−5·1–35·7	−10·8–36·3	3·8–33·4	−4·0–42·6	−8·3–39·3	0·23–45·0

	Day 0↓			Day 28		
Immunoglobulins	IgA	IgM	IgG	IgA	IgM	IgG
BCG (n.s.)	2·60	1·25	13·7	2·85	1·55	17·0
	1·5–4·1	0·4–1·7	9·0–21·5	2·3–3·9	0·3–1·8	10·0–24·0
C. parvum (n.s.)	1·60	1·35	11·75	2·00	1·25	13·60
	1·0–5·3	0·5–1·7	6·5–17·5	1·0–6·1	0·5–3·0	5·3–15·5

* For explanation of symbols see footnotes to Table 2.

TABLE 4. Phytohaemagglutinin blastogenesis (medians, ranges and statistical differences)

Day	0↓	(3 hr)	2	4	7	10	14	21	28
Spontaneous uptake ^{3}H-thymidine (ct/min)									
BCG‡	590	—	906	948	1131	1441§	1156§	1157§	992§
	251–1833	—	610–1720	444–1754	479–2425	1080–4166	944–3648	919–5071	360–3536
C. parvum (n.s.)	1115	943	1146	1198	1632	1366	1706	1392	1072
	273–4417	191–2963	303–2033	196–3217	212–2638	172–2256	830–2991	826–3409	847–1702
Maximum uptake ^{3}H-thymidine with PHA (ct/min)									
BCG‡	28264	—	22356	21181	26003	53324§	53762§	88036§	30194
	13599–61625	—	13158–47543	6477–57543	12002–99270	40387–141409	43204–121301	40877–144400	14928–76054
*C. parvum**	23964	15709	34303	46008	59948§	96619§	62062	43404	34299
	4291–140931	945–126939	5163–133281	5242–125752	11211–250138	11365–216804	37186–147456	20420–122566	16943–149324
Stimulation index									
BCG (n.s.)	37·0	—	23·0	23·4	22·8	35·1	40·2	44·3	28·8
	23·8–118·3	—	12·7–43·7	8·0–50·6	11·5–62·9	9·5–55·9	27·1–60·4	27·5–71·0	15·3–88·8
*C. parvum**	24·9	15·7	26·5	37·0§	44 4§	45·9	34·6	29·7	27·4
	6·0–64·2	4·0–68·3	16·0–64·6	14·4–65·5	15·4–144·9	19·2–192·9	22·5–86·5	14·5–123·3	10·1–153·4
Log_{10} mitogenic capacity									
BCG‡	7·518	—	7·387	7·572	7·589	7·991§	7·979§	8·009§	7·847
	7·208–8·027	—	6·861–7·876	6·942–7·939	7·129–8·445	7·608–8·574	7·377–8·426	7·693–8·627	7·137–8·179
C. parvum†	7·613	6·965§	7·266	7·725	8·140	8·280	8·052	8·029	8·032
	6·979–8·449	6·193–7·871	6·231–7·781	6·853–8·212	7·237–8·826	7·127–8·678	7·758–8·438	7·396–8·472	7·175–8·496
Log_{10} optimal adjusted response									
BCG‡	4·969	—	4·857	4·843	4·931	5·261§	5·246§	5·475§	4·997
	4·652–5·346	—	4·638–5·195	4·330–5·279	4·598–5·515	5·152–5·669	5·154–5·640	5·130–5·678	4·693–5·400
C. parvum (n.s.)	4·898	4·713	5·054	5·177	5·271	5·508	5·311	5·149	5·035
	4·151–5·688	3·494–5·622	4·232–5·643	4·238–5·636	4·568–5·917	4·612–5·855	5·089–5·687	4·829–5·607	4·748–5·695

* For explanation of symbols see footnotes to Table 2.

Changes in immunological profile following immunization with BCG or C. parvum

The results of the Friedman analyses for overall change and, where significant, the results of Wilcoxon analyses are given in Tables 2, 3 and 4. Early decreases in median values for all parameters except the percentage of null cells were noted after *C. parvum* (at 3 hr, up to 7 days); these were statistically significant for the white count, lymphocyte count, E-rosette and sIg counts and for the mitogenic capacity. Similar decreases in median values were noted after BCG, but were less prolonged and not statistically significant.

Recovery occurred by day 4 or 7 and increases above pre-immunization values followed. The increases in median values for the various assays were maximal in the 7–21 day interval and declined towards initial values by day 28. The increases were statistically significantly different from the 'pre-*C. parvum*' immunization values for the E-rosette cell count, and PHA blastogenesis expressed as maximum uptake and stimulation index, but not when calculated using the OAR method. After BCG, significant increases were found in the percentages of E-rosettes, sIg cells (with a corresponding decrease in the percentage of null cells and null cell count) and all PHA blastogenesis expressions, except the stimulation index.

DISCUSSION

Analysis of the sequential data was complicated by 'non-normal' distribution in the assay values, multiple sequential assays and the large volume of information generated. A computer programme was designed to overcome these problems.

The reduction in T cells observed in the patients before immunization confirms other reports (Wybran & Fudenberg, 1976; Golub, O'Connell & Morton, 1974). Immunosuppression or the inability of the immune system to react to a stimulus could be an explanation for the failure of monitoring to detect changes in some immunotherapy studies. However, this suggestion is not supported by the present investigation in which statistically significant decreases and increases were observed after immunization.

The early decrease in the white cell and lymphocyte count after *C. parvum* administration has been described using higher doses 5·0 mg/m^2 than those reported in this study (Gill *et al.*, 1978). A significant reduction in T and sIg$^+$ cell numbers together with a reduction in PHA blastogenesis, also occurred in the first week after *C. parvum*, a similar trend (non-significant) was noted after BCG. These changes have not been previously described in man.

After recovery, increases in median values (above pre-immunization values) occurred during the second and third weeks. These were statistically significant for T cell numbers (and blastogenesis) after *C. parvum*, and also for sIg$^+$ cell percentage after BCG. Other authors have found no change in these parameters after *C. parvum* in breast carcinoma (Minton *et al.*, 1976) nor after BCG in bronchial carcinoma (Oldham *et al.*, 1976). In the present study, the patient groups were well defined with similar clinical features and performance status, there was also no obvious increase in tumour burden during the study period. These factors and the strict collection of all samples on multiple occasions over a short time course and the use of fresh lymphocytes may have assisted the detection of significant change. The increases in immunological parameters were declining to base line values during the fourth week; monthly assays (Oldham *et al.*, 1976; Golub, Forsythe & Morton, 1977) would not detect any increases if the immunization schedule was ineffective at maintaining increased reactivity.

Cells carrying surface immunoglobulin were represented by a somewhat higher proportion of lymphocytes than is currently considered to be of B cell origin. A proportion of cells with adsorbed IgG might have been responsible and this uncertainty requires that these cells be described as sIg$^+$ cells. These data and that of the null cells (which may have been underestimated) are included for completeness and as there is a paucity of information describing the effects on these two subpopulations of immunotherapy at present.

It may be more relevant when measuring changes in immunity to calculate results related to the numbers of circulating lymphocytes (e.g. the percentage of E-rosettes expressed as 'absolute' numbers). The mitogenic capacity represents the PHA responsiveness in 1·0 ml of blood (Campbell *et al.*, 1973).

The optimal adjusted response, OAR, takes into account responses at low PHA concentrations, which may be more sensitive to change, than the response at optimal PHA concentration; in addition, more data describing the PHA titration curve are utilized (Gross & Eddie-Quartey, 1976). After BCG therapy, the spontaneous and maximum uptakes, mitogenic capacity and OAR all increased. But with *C. parvum*, the OAR did not increase significantly, although the stimulation index and mitogenic capacity did rise significantly. This result fails to support the 'increase in sensitivity' claimed for the OAR compared with other expressions. The pattern of response (decline, recovery, overshoot, etc.) with time indicates the need for analysis at different time intervals following immunization. Grouping of data at different time intervals might have resulted in no significant differences being observed. Furthermore, reports of differences in immune status following immunization would be of more clinical use if values at various intervals were documented.

Major surgery is known to result in transient depression of T and B cells but with complete recovery by day 5 to 7 (Slade *et al.*, 1975; Kehlet *et al.*, 1977). It is difficult to separate the effect of trauma with resultant glucocorticoid release from the effect of general anaesthesia. The early immunodepression that followed *C. parvum* therapy could have resulted from a stress reaction to the pyrexia associated with this agent. However, this depression was marked by as early as 3 hr, whilst the side effects of *C. parvum* were usually absent until 5–6 hr had elapsed. Moreover, BCG administration, which was without symptomatic side effects was also associated with an early immunodepressive trend. Direct clarification of the role of stress would require saline administrations to a control group of untreated melanoma patients. This might be considered unjustifiable.

The changes in lymphoid subpopulations will also be discussed in the following report describing the cytotoxicity data.

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