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A Comparative Study of Anaerobic Coryneforms

ATTEMPTS TO CORRELATE THEIR ANTI-TUMOUR ACTIVITY WITH THEIR SEROLOGICAL PROPERTIES AND ABILITY TO STIMULATE THE LYMPHORETICULAR SYSTEM

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Summary. Various strains of anaerobic coryneforms and the closely related *Propionibacteria* have been compared *in vivo* with respect to their anti-tumour activity. Their effectiveness has been correlated with their serological relationship and to some extent with their ability to stimulate the lymphoreticular system. Organisms belonging to *Corynebacterium acnes* groups I and II and *C. avidum* group IV were active anti-tumour agents, although of varying effectiveness. These strains are serologically closely related and all produce a soluble cross-reacting antigen. The single *C. granulosum* group III strain which we tested, an unclassified coryneform, and the classical *Propionibacteria* did not cross-react with the main group and had little or no anti-tumour activity. At the high dose (0·7 mg) we used, all strains, whether they inhibited tumour development or not, enhanced clearance of colloidal carbon and stimulated production of an inflammatory peritoneal exudate; at lower dosage the results were too variable to permit valid comparison. At the higher dose anti-tumour activity of a strain appeared to correlate best with ability to produce splenomegally and decrease red cell volume in the blood.

INTRODUCTION

In recent years certain anaerobic coryneforms, in particular *Corynebacterium parvum*, have been extensively used as anti-tumour agents following the discovery that in mice killed preparations stimulate cells of the reticuloendothelial system (Halpern, Prévot, Biozzi, Stiffel, Mouton, Morard, Bouthillier and Décreusefond, 1964) and inhibit the growth of isogeneically transplanted tumours (Woodruff and Boak, 1966; Woodruff and Dunbar, 1973).

Further investigations on the mechanism of action of these micro-organisms have been hampered by insufficient knowledge of their bacteriological identity. Perhaps as a result, there have been few comparative studies of the biological activities of the anaerobic coryneforms and their components. Recently, however, Johnson and Cummins (1972) have compared the bacteriology of these and related micro-organisms, and some of their

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biological properties have been described (O'Neill, Henderson and White, 1973; Wilkinson, O'Neill and Wapshaw, 1973). In this study, we have concentrated on the ability of these organisms to inhibit tumour growth and have attempted to correlate this with their effect upon the lymphoreticular system and with certain biochemical features of the organisms.

MATERIALS AND METHODS

Bacteria

Organisms were obtained from the National Collection of Type Cultures, Colindale (NCTC), the American Type Culture Collection (ATCC), and Professor C. S. Cummins (Virginia Polytechnic and State University, Blacksburg, Virginia) as follows: *Corynebacterium parvum* strains 10390 (NCTC), 10387 (NCTC) and 0208 (Cummins); *C. acnes* strains 737 (NCTC) and 11828 (ATCC); *C. anaerobium* 0162 (Cummins); *Propionibacterium avidum* 4982 (Cummins) and *P. freudenreichii* 10470 (NCTC). A formalin-killed preparation of *C. parvum* CN6134 was a kind gift from Burroughs Wellcome Laboratories, Beckenham, Kent.

It has been suggested (Douglas and Gunter, 1946; Johnson and Cummins, 1972) that all these organisms, and others related to them, should be placed in the genus *Propionibacterium* rather than *Corynebacterium.* In this paper we have retained the more classical nomenclature as we feel that the weight of evidence, including that presented here, is in its favour. However, in all other respects we accept, and use, the subdivision of coryneforms proposed by Johnson and Cummins (1972).

The bacteria were grown semianaerobically at 37° in horse digest broth +3 per cent glucose. They were harvested in stationary phase, killed with formalin (0·5 per cent), washed in saline and adjusted to 7 mg/ml dry weight.

Unless otherwise stated all animals received 0·1 ml of this preparation intraperitoneally.

Soluble bacterial extracts

Supernatants from bacterial cultures were used as sources of soluble antigen.

Serology

Antigenic relationships between the whole organisms were examined using the agglutination test described elsewhere (Woodruff, McBride and Dunbar, 1974).

Soluble antigen was detected by counterimmunoelectro-osmophoresis (CIEOP) according to the method of Prince and Burke (1970) except that veronal buffer of ionic strength 0·033 and pH 8·2 was used. Its ability to bind to sheep red blood cells was assayed by passive haemagglutination as described by McBride, Jones and Weir (1974).

Animals

Adult female CBA mice (18–25 g) were used in all experiments.

Tumour

The tumour used was an isogeneic methylcholanthrene-induced fibrosarcoma in its fifteenth transplant generation. For *in vivo* experiments five to eight mice were inoculated subcutaneously with 10^4 viable tumour cells prepared by pronase treatment (Woodruff

and Boak, 1966). We have taken as the index of tumour growth the sum of individual tumour diameters measured every 2 days up to a set time (Woodruff, Dunbar and Ghaffar, 1973).

In vitro cytostasis of the tumour cells by lymphoid cells was assessed by using ^{125}I-labelled UdR incorporation to measure the number of proliferating tumour cells 2 days after incubation. This technique has been described elsewhere (Ghaffar, Cullen, Dunbar and Woodruff, 1974). Pools of lymphoid cells from one to three mice were used per experiment.

Carbon clearance

The phagocytic index, based on the rate of clearance of intravenously injected colloidal carbon, was determined by an adaptation of the method of Biozzi, Benacerraf, Stiffel and Halpern (1954) described by Woodruff, McBride and Dunbar (1974) although K values are expressed omitting the multiplication factor of 100.

Measurement of splenic hypertrophy

Mice were killed by ether inhalation and the spleens removed for immediate weighing.

Collection and examination of peritoneal exudate cells

Peritoneal exudate cells were collected by peritoneal lavage with 2·5 ml Dulbecco's solution + 10 i.u. of heparin/ml. Total and differential white cell counts were performed on all preparations.

Packed cell volume

Blood samples were taken from the retro-orbital sinus into heparinized micro-haematocrit tubes. These were centrifuged for 15 minutes at 3000 ***g*** and the packed cell volume was measured as a percentage of total blood volume.

RESULTS

ANTIGENIC RELATIONSHIPS BETWEEN ANAEROBIC CORYNEFORMS AND THEIR ABILITY TO PRODUCE SOLUBLE ANTIGEN

The anaerobic coryneforms have been divided into four taxonomic groups on the basis of serology, cell wall composition and DNA homology (Johnson and Cummins, 1972). However, Table 1 (column 1) shows that antigens are shared between organisms classed as belonging to groups I, II and IV. This was first detected by agglutination tests using rabbit antisera. Although the results in Table 1 only refer to antisera to *C. parvum* 6134 (group I), identical results were obtained with antisera to 737 (group I) and 10390 (group II). Organisms that did not agglutinate with these antisera were the classical *Propionibacteria*, *C. parvum* 10387 (group III) and the coryneform 7371, which was derived from a culture of *C. acnes* 737.

Media from fresh stationary phase cultures of organisms of the main cross-reacting homology groups I, II and IV contained considerable quantities of soluble cross-reacting antigen as detected by counterimmunoelectro-osmophoresis (CIEOP). Like the whole organisms, this reacted with antisera raised against organisms from either group I or group II (Table 1, column 2). Most antigen preparations gave two or more precipitin lines

TABLE 1

ANTIGENIC RELATIONSHIPS BETWEEN ANAEROBIC CORYNEFORMS AND PROPIONIBACTERIA, AND PRODUCTION OF SOLUBLE ANTIGEN

Group	Strain	Number	Agglutination with anti-*C. acnes* I (log_2 Ab titre)	Quantity of soluble antigen (log_2 Ag titre)		Absorbed anti-10390 *vs* 10390 (log_2 Ab titre)*	
				CIEOP	Passive HA	Agglutination	CIEOP
C. acnes I	*C. parvum*	6134	11	(+)	(+)†	5	0
	C. parvum	0208	9	8	3	5	0
	C. acnes	737	10	7	6	0	0
	C. acnes	6922	7	6	2	7	0
	C. acnes	11827	11	6	2	5	0
C. acnes II	*C. parvum*	10390	10	8	4	5	0
	C. acnes	11828	10	8	3	6	0
	C. acnes	0147	10	6	2	5	0
	C. anaerobium	0162	10	8	3	5	0
C. granulosum III	*C. parvum*	10387	5	0	0	10	4
C. avidum IV	*P. avidum*	4982	10	2	1	6	1
'Classical'	*P. rubrum*	4872	0	0	0	9	2
Propionibacteria	*P. freudenreichii*	10470	0	0	0	10	4
	Coryneform contaminant	7371	3	0	0	10	4
Controls			0–4	0	0	10	4

* Antiserum against strain 10390 was absorbed with an equal volume of packed bacterial cells of different strains at 37° for 1 hour before testing against strain 10390.

† This strain was only available as a killed suspension; suspension medium rather than growth medium was therefore tested for soluble antigen.

of varying strengths on immunodiffusion but all gave reactions of identity where this could be tested. No soluble antigen could be detected in cultures of organisms that did not cross-react with the main *C. acnes* group, even when antisera raised against *C. parvum* 10387 and *P. freudenreichii* 10470 were used.

All culture media containing soluble antigen also had antigen that could bind to the surface of sheep red cells as detected by passive haemagglutination (Table 1, column 2).

Antisera against strain 10390 were absorbed with the different organisms, and the results confirmed that there was a main cross-reacting homology group (Table 1, column 3).

ANTI-TUMOUR ACTIVITY OF DIFFERENT CORYNEFORMS

The same preparations of the different strains of coryneforms that were tested above were examined for their ability to inhibit tumour growth. The bacterial suspensions (0·7 and 0·35 mg) were injected intraperitoneally into CBA mice 3 days after subcutaneous injection of 10^4 fibrosarcoma cells. The results are shown in Table 2. As reported previously (Woodruff and Dunbar, 1973), 0·7 mg and 0·35 mg of CN6134 gave significant inhibition of tumour growth. At 0·7 mg, but not at the lower dose (0·35 mg), strain 4982 was marginally more effective. Strain 10390 also significantly inhibited tumour growth at the higher dose (0·7 mg). Slight inhibition of tumour growth was seen with all other strains except 10387 and 7371 which were totally inactive.

Inhibition of tumour cell proliferation *in vitro* was investigated using peritoneal exudate and spleen cells from mice injected 4 days previously with 0·7 mg of the different strains of organisms. The results, set out in Table 3, show a good correlation with the *in vivo*

TABLE 2

ANTI-TUMOUR ACTIVITY OF DIFFERENT ANAEROBIC CORYNEFORMS (*in vivo*)

Organism		Dose	Number	Sum of tumour diameters (mm)†			
Group	Strain number	(mg)*	of mice	Observed values	$\bar{X}$‡	*t*s.e.‡	*P*‡
—	—	—	13	76, 77, 29, 88, 78, 82, 62, 69, 67, 90, 95, 70, 85.	74·5	9·9	
C. acnes I	6134	0·7	6	45, 41, 48, 54, 28, 51.	44·5	9·8	<0·001
C. acnes I	0208	0·7	3	57, 40, 64.	53·7	30·5	<0·1
C. acnes II	10390	0·7	6	48, 32, 56, 49, 50, 53.	48·0	8·7	<0·002
C. acnes II	11828	0·7	5	62, 48, 37, 63, 46.	51·2	13·9	<0·02
C. acnes II	0162	0·7	6	52, 54, 62, 57, 56, 40.	53·5	7·7	<0·01
C. granulosum	10387	0·7	6	93, 82, 81, 67, 80, 81.	80·7	8·7	n.s.
C. avidum	4982	0·7	6	38, 44, 47, 45, 20, 0.	32·3	19·5	<0·001
Propionibacterium	10470	0·7	7	68, 58, 58, 53, 58, 77, 51.	60·4	8·3	<0·1
Coryneform	7371	0·7	7	90, 74, 79, 88, 70, 68, 63.	76·0	9·0	n.s.
C. acnes I	6134	0·35	7	48, 57, 21, 37, 39, 24, 39.	36·4	12·0	<0·01
C. acnes II	10390†	0·35	7	53, 51, 61, 77, 60, 53, 45.	57·1	9·5	<0·05
C. avidum	4982	0·35	7	77, 59, 28, 49, 42, 55, 70.	54·3	15·4	<0·05

n.s. = Not significant.

* Bacteria were injected intraperitoneally.

† Individual tumour diameters were measured at intervals and the sum of these values used as an index of tumour growth.

‡ The significance of the results (*P* values) was determined by the standard two-tail *t*-test method, $\bar{X}$ denotes group mean sum, *t* s.e. denotes Student's function *t* × standard error.

TABLE 3

ANTI-TUMOUR ACTIVITY OF DIFFERENT ANAEROBIC CORYNEFORMS (*in vitro*)

Organism		Number	Spleen cells			Peritoneal cells		
Group	Strain number	of cultures	Ratio	CI*	*P*†	Ratio	CI*	*P*†
—	—	5	400:1	0	—	40:1	0	—
		5	200:1	0	—	20:1	0	—
		5	100:1	0	—	10:1	0	—
C. acnes I	6134	4	400:1	94	<0·01	40:1	97	<0·01
		4	200:1	78	<0·01	20:1	96	<0·01
		4	100:1	24	<0·05	10:1	80	<0·01
C. acnes II	10390	5	400:1	76	<0·01	40:1	20	<0·05
		5	200:1	12	n.s.	20:1	18	<0·05
		5	100:1	−81	<0·01	10:1	−7	n.s.
C. granulosum III	10387	4	400:1	43	<0·05	40:1	15	n.s.
		4	200:1	−58	<0·01	20:1	25	<0·05
		4	100:1	−8	n.s.	10:1	25	<0·05
C. avidum IV	4982	4	400:1	91	<0·01	40:1	85	<0·01
		4	200:1	41	<0·01	20:1	39	0·01
		4	100:1	−62	<0·01	10:1	7	n.s.
Propionibacterium	10470	4	400:1	39	<0·01	40:1	6	n.s.
		4	200:1	−22	<0·1	20:1	5	n.s.
		4	100:1	−52	<0·1	10:1	−14	n.s.
Coryneform	7371	5	400:1	−2	n.s.	40:1	−11	n.s.
		5	200:1	−100	<0·01	20:1	2	n.s.
		5	100:1	−24	n.s.	10:1	−34	n.s.

n.s. = Not significant.

* CI = cytostatic index.

† The significance of the results (*P* values) were determined by the standard two-tail *t*-test method.

tumour experiments. As discussed elsewhere, negative values suggest stimulation of cell proliferation (Fidler, 1973). Strain 6134 was highly effective in stimulating cytotoxic cells at all effector:target cell ratios tested. Strains 4982 and 10390 stimulated effector cells which were cytostatic at high effector:target cell ratios whereas, by comparison, 10387, 7371 and 10470 had little effect.

OTHER BIOLOGICAL ACTIVITIES OF THE CORYNEFORMS

One of the most striking effects of the anaerobic coryneforms is their ability to stimulate cells of the reticuloendothelial system (Halpern *et al.*, 1964). To investigate the relationship of this to an anti-tumour effect, groups of six CBA mice were injected intraperitoneally with the different strains of coryneforms and their spleens weighed 10 days later. The packed cell volume (PCV), which is probably closely linked to splenomegaly (McBride,

TABLE 4

EFFECT OF DIFFERENT ANAEROBIC CORYNEFORMS ON PACKED CELL VOLUME AND SPLEEN WEIGHT

Organism		Packed cell volume (day 10)*			Spleen weight (day 10)*		
Group	Strain number	Per cent	*t.* s.e.†	*P*†	mg	*t.* s.e.†	*P*†
—	—	49	0·9	—	49	9	—
C. acnes I	6134	34	4·5	<0·01	328	29	<0·01
C. acnes I	0208	41	7·4	n.s.	307	32	<0·01
C. acnes II	10390	33	1·9	<0·01	324	39	<0·01
C. acnes II	11828	32	4·3	<0·01	311	109	<0·01
C. acnes II	0162	36	0·6	<0·01	291	50	<0·01
C. granulosum III	10387	47	1·9	n.s.	67	19	n.s.
C. avidum IV	4982	33	2·5	<0·01	353	77	<0·01
Propionibacterium	10470	38	3·1	<0·01	85	31	<0·05
Coryneform	7371	47	1·2	n.s.	77	11	<0·01

n.s. = Not significant.

* Values measured 10 days after i.p. injection of bacteria.

† The significance of the results (*P* values) were determined by the standard two-tail *t*-test method, *t.* s.e. denotes Student's function $t \times$ standard error.

TABLE 5

ENHANCED CARBON CLEARANCE AND PERITONEAL EXUDATE POPULATION AFTER INJECTION OF DIFFERENT ANAEROBIC CORYNEFORMS

Organism		Phagocytic index (day 4)			Phagocytic index (day 10)			PEC × 10^6 (day 4)‡		
Group	Strain number	*K**	*t.* s.e.†	*P*†	*K**	*t.* s.e.†	*P*†	Number	*t.* s.e.†	*P*†
—	—	0·02	0·006	—	0·01	0·004	—	3·1	0·71	—
C. acnes I	6134	0·07	0·01	<0·01	0·04	0·02	<0·01	5·9	1·69	<0·01
C. acnes II	10390	0·06	0·01	<0·01	—	—	—	3·9	0·93	<0·1
C. granulosum III	10387	0·08	0·01	<0·01	0·07	0·05	<0·01	5·8	0·61	<0·01
C. avidum IV	4982	0·08	0·02	<0·01	0·05	0·02	<0·01	3·9	1·29	n.s.
Propionibacterium	10470	0·10	0·04	<0·01	—	—	—	6·0	1·25	<0·01
Coryneform	7371	0·08	0·02	<0·01	0·04	0·01	<0·01	7·3	1·76	<0·01

n.s. = Not significant.

* K = phagocytic index.

† The significance of the results (*P* values) were determined by the standard two-tail t-test method, *t.* s.e. denotes Student's function $t \times$ standard error.

‡ PEC denotes total peritoneal exudate white cell count.

TABLE 6

SUMMARY OF RESULTS

Organism		Cross-reactivity	Anti-tumour activity		Splenomegaly	Decrease in PCV*	Phagocytic index	Increase in PEC
Group	Strain number		*In vivo*	*In vitro*				
C. acnes I	6134	++	+++	+++	+++	+++	+	+
C. acnes I	0208	++	++	n.d.	+++	+	+	n.d.
C. acnes II	10390	++	+++	++	+++	+++	+	±
C. acnes II	11828	++	++	n.d.	+++	+++	n.d.	n.d.
C. acnes II	0162	++	++	n.d.	+++	++	n.d.	n.d.
C. granulosum	10387	−	−	±	−	−	+	+
C. avidum	4982	++	+++	+++	+++	+++	+	±
Propionibacterium	10470	−	±	±	±	++	+	+
Coryneform	7371	−	−	−	±	−	+	+

n.d. = Not done.

* PCV = Packed cell volume.

† PEC = total peritoneal exudate white cell count.

Jones and Weir, 1974), was also measured at this time. The results are set out in Table 4. It can be seen that there is a fairly good correlation between a drop in packed cell volume, splenomegaly and the anti-tumour activity of the organisms. The most effective organisms were strains 6134, 4982 and 10390. Strains 10387, 10470 and 7371 were relatively ineffective.

We also examined the effect of the different strains of organisms on clearance of colloidal carbon from the blood, and their capacity to induce an inflammatory peritoneal exudate. At a dose of 0·7 mg all strains were found to increase the phagocytic index either 4 days or 10 days after injection to about the same extent (Table 5). Moreover, there was no correlation between their anti-tumour activity and the extent of the inflammatory peritoneal exudate induced after four days.

At lower dosage (0·35 and 0·175 mg) the effect on phagocytic index was variable with two of the three organisms tested (strains 10387 and 7371), which show weak or absent anti-tumour effect, as compared with strain CN6134 which has marked anti-tumour activity.

A summary of all the results is given in Table 6.

DISCUSSION

Until recently a major problem in comparing the biological effects of different strains of anaerobic coryneforms has lain in establishing the taxonomic identity and relationships of many of the organisms. This has been partly alleviated by Johnson and Cummins (1972), who divided the strains into four groups on the basis of serology, cell wall composition, and estimates of DNA homology. Recent comparative studies have used this classification to good effect (O'Neill *et al.*, 1973; Wilkinson *et al.*, 1973).

In this study we have shown that coryneforms of all the groups, with the possible exception of group III, appear to be serologically closely related and are antigenically distinct from the classical *Propionibacteria* (Table 6).

All strains of the serologically related groups of coryneforms produce a soluble cross-reacting antigen towards the end of the growth cycle. It is antigenic as detected on counter-immunoelectro-osmophoresis using antisera to any of the related organisms. Moreover, it binds to red blood cells as detected by passive haemagglutination using the same antisera. As reported elsewhere (Dawes, Tuach and McBride, 1974), it is an acidic polysaccharide and clearly represents a major cell wall component.

As an initial attempt to relate structural and bacteriological properties of the coryneforms to their anti-tumour effectiveness, those strains of bacteria which had been compared serologically were examined for biological activity. In the system used, the three strains 6134, 4982 and 10390 were particularly active as inhibitors of tumour growth *in vivo*. They belong to the cross-reacting groups I, IV and II respectively. The other members of these groups that we tested were also effective but to a lesser extent. Only two organisms, 10387 and 7371, had no effect on tumour growth *in vivo*. Woodruff and Dunbar (1973) have previously reported some anti-tumour activity with strain 10387 at a higher dose than that used here. However, this strain is apparently mixed (O'Neill, Henderson and White, 1973), and the discrepancy is probably the result of the preponderance of different organisms in different batches. A single batch of 10387 was used throughout the work reported in this paper; the results are therefore internally consistent though not necessarily comparable with other experiments using the same designated strain. Neither this batch of 10387, nor 7371, the other ineffective organism, was serologically similar to the active group, and the other non-cross-reacting organism tested, *P. freudenreichii*, displayed only

minimal anti-tumour activity. Although the strains vary in their activity, it would appear from our results, that to be effective an anaerobic coryneform may have to belong to the main serologically cross-reacting groups (Table 6). Identical conclusions can be drawn from the results of the *in vitro* tumour assay.

A comparison of other biological properties of the different strains was carried out in an attempt to locate a cellular response to the bacteria which may be the mediator of the anti-tumour effect.

The ability of the different strains to produce splenomegaly and decrease blood packed cell volume 10 days following injection appeared to correlate well with their anti-tumour effect.

In contrast, all strains tested increased the rate of clearance of colloidal carbon from the blood stream when measured either 4 or 10 days later. This differs from other published results (O'Neill *et al.*, 1973; Woodruff and Dunbar, 1973), in which different doses were used. As Woodruff and Dunbar (1973) have found, the phagocytic index, unlike splenomegaly, is highly dependent upon the dose of organisms, which may perhaps explain the discrepancy. Nor was there any correlation at a dose of 0·7 mg between the anti-tumour activity of a particular strain and its ability to induce an inflammatory peritoneal exudate 4 days later, if anything the reverse, although we have not investigated the possibility that cells could be lost from the peritoneal cavity at this time, as occurs following administration of endotoxin or double-stranded RNA (Parr, Wheeler and Alexander, 1973).

At present we do not know how the 'active' coryneforms stimulate tumour immunity. In our system we have evidence that the macrophage may be important, as *C. parvum* is an effective anti-tumour agent in T-cell deprived mice (Woodruff, Dunbar and Ghaffar, 1973). In addition glass-adherent cells seem to be responsible for tumour cell killing *in vitro* (Ghaffar *et al.*, 1974). The way in which these active cells are generated is also unknown. A pre-existing state of immunity to *C. parvum* may be a pre-requisite (Woodruff *et al.*, 1974), or alternatively the bacteria may stimulate the macrophages directly. As yet it would be premature to speculate on a role for the soluble, cell-attaching, polysaccharide antigen. Wilkinson *et al.* (1973) have shown that these organisms can produce an as yet unidentified product which can stimulate serum-independent macrophage chemotaxis. It would be of interest to know if this were the same as the soluble antigen.

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