BIOLOGICAL EFFECTS OF CORYNEBACTERIUM PARVUM: III. AMPLIFICATION OF RESISTANCE AND IMPAIRMENT OF ACTIVE IMMUNITY TO MURINE TUMOURS

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Summary.—The effect of pre-treatment with Corynebacterium parvum on the growth in vivo of a range of experimental mouse tumours with differing characteristics has been investigated. Varying degrees of protection were observed which were generally greater with the more immunogenic tumours. Administration of C. parvum 7 days before immunization with irradiated tumour cells diminished the protective effect which could be obtained by immunization alone. The possible basis for these seemingly conflicting influences is considered.

Halpern et al. (1964) showed that a killed suspension of Corynebacterium parvum was an unusually potent stimulant for the reticulo-endothelial (macrophage) system. A number of associated effects such as adjuvant activity (Neveu, Brellec and Biozzi, 1964; Biozzi et al., 1966) and increased resistance to bacterial (Adlam, Broughton and Scott, 1972) and protozoal (Nussenzweig, 1967) infection, have since been reported. The inhibitory effect of C. parvum pre-treatment on the growth of a range of experimental mouse tumours (Sarcoma 1, Ehrlich ascites, a spontaneous mammary carcinoma, a methylcholanthrene-induced sarcoma and the AKR leukaemia) has been described (Halpern et al., 1966; Woodruff and Boak, 1966; Lamensans et al., 1968). Although the results obtained here varied between the different systems, in general, conditions could be found under which C. parvum afforded a degree of protection. Currie and Bagshawe (1970) used a combination of C. parvum and chemotherapy against a methylcholanthrene-induced fibrosarcoma with some success, whereas Mathé, Pouillart and Lapeyraque (1969) were unable to influence an established L1210 tumour with a combination of C. parvum and immunotherapy.

The experiments described here are concerned with two aspects of the anti-tumour activities of C. parvum: the effect of simple pre-treatment and of pre-treatment followed by active immunization on the growth of a primary tumour challenge. Several mouse tumour systems, providing a variety of growth patterns and immunogenicities, have been used and the relationship between the effects of C. parvum and the characteristics of the tumours are discussed. The investigation formed a prelude to studies at the cell level and tissue culture lines have been established from the tumours described, in order to facilitate subsequent work in vitro.

MATERIALS AND METHODS

Mice.—Adults of the following inbred strains and F1 hybrids, maintained in this Department, were used: CBA-p (from the Department of Genetics, University of Cambridge), DBA/2 and BALB/c (from the Chester Beatty Research Institute) and (BALB/c × DBA/2)F1. Groups of 10 mice were used for each experimental group throughout.

Corynebacterium parvum.—A killed suspension of C. parvum (Batch No. EZ174 7 mg/
ml) was provided by Wellcome Research Laboratories, Beckenham, Kent, England. A standard dose of 0.2 ml (1.4 mg) was injected i.v. or i.p.

Tumours.—The following tumours were obtained from the Chester Beatty Research Institute and maintained in ascitic form in the mice specified: R—I (radiation induced CBA leukaemia; Hewitt, 1962) in CBA—p; Hepatoma 129 (induced by CCl₃ in a C3H mouse, Andervont and Dunn, 1955) in CBA—p and BALB/c; Adj. PC6A (adjuvant induced BALB/c plasmacytoma, Potter and Robertson, 1960) in BALB/c and L5178 (DBA/2 leukaemia, Fischer, 1958) in (BALB/c × DBA/2)F₁,

The CBAT-3 fibrosarcoma was derived originally from a tissue culture line of CBA embryo fibroblasts and maintained as a subcutaneous solid tumour in CBA—p mice.

For both routine passage and experimental use, tumours were handled as cell suspensions in phosphate buffered saline. Ascites cells were harvested direct and washed once. Solid tumours were dissociated by mincing with scissors and pipetting the fragments. The supernatant cells, after settling, were washed once.

Inactivation of tumour cells.—Cells were inactivated by in vitro irradiation (15,000 rad) from a ⁶⁰Co source. This procedure did not affect the viability of the cells as judged by trypan blue exclusion.

Assessment of results.—Results are displayed as curves of percentage of survivors against days after challenge. Animals whose deaths are not recorded were kept for 60 days. In the case of the subcutaneous CBAT-3 tumour, a 2 cm diameter was taken as the endpoint if it occurred before death.

RESULTS

Characteristics of the tumours and their immunogenicity

Immunogenicity of the various tumours was assessed by the protective effect of immunization with inactivated tumour cells 10 days before challenge with normal tumour cells. Immunization against ascitic tumours was i.p., but for solid tumours the immunizing dose was split between the i.p. route and a subcutaneous (s.c.) site contralateral to that of the challenge.

RI leukaemia cells are syngeneic in CBA mice and grow rapidly, causing death from incula of fewer than 10 cells. Death is abrupt with only moderate ascites development, suggesting that metastasis is a major factor. The tumour as first grown here was moderately immunogenic in CBA—p mice, a single dose of 10⁸—5 × 10⁵ irradiated cells giving 50% protection against a 100 cell challenge. This form of the tumour is referred to as RI. Weekly passage through mice for 18 months resulted in a variant form which was very much less immunogenic (RI-var.); 5 × 10⁵ irradiated RI-var. gave almost no protection against a 100 cell challenge. Recourse to frozen stored material allowed comparisons between the original and variant forms.

Hepatoma 129 grows rapidly from inocula of less than 10 cells and causes death with gross ascites and no evidence of metastases. Our form is not strain specific; originally a C3H tumour, it is routinely passaged in CBA against a minor histocompatibility barrier and a secondary strain is passaged in BALB/c mice against a strong histocompatibility barrier. There is little difference between the growth rate or minimum lethal dose of the two strains. The immunogenicity of irradiated cells is also similar, a dose of between 10⁴ and 5 × 10⁴ giving 50% protection against a 100 cell challenge. The lack of strain specificity is not due to masking or loss of histocompatibility antigens, since BALB/c mice were readily immunized against the tumour by normal CBA spleen cells.

CBAT-3 fibrosarcoma grows as a non-metastasizing solid when injected s.c. Its liability to become haemorrhagic or to break through the skin makes quantitative assessment difficult. When injected i.p. it grows more rapidly as localized solid tumours in the peritoneal wall and consistently causes death in the animals.

Assessment of the immunogenicity of irradiated cells depends on the route of injection of challenge cells. Protection
is low against s.c. challenge with $10^4$ cells, $10^6-5 \times 10^6$ irradiated cells being required to give 50\% protection. The titration with i.p. challenge did not reach an endpoint but protection was at least 10 times greater.

*L5178 leukaemia* causes death essentially by massive i.p. growth with some local infiltration to form solid mesenterial growth. The F$_1$ hybrid in which the tumour was passaged was within the major histocompatibility group (H–2$^d$) of the strain of origin (DBA/2), thus minimizing any potential effects of allogeneic inhibition. The tumour was poorly immunogenic in this system and up to $10^7$ irradiated cells were required for 50\% protection against a 100 cell challenge.

*PC6 plasmacytoma* gives rise to massive ascites, with ready formation of a solid tumour at the injection site; it shows no evidence of metastasis. It is poorly immunogenic, with no protection against a $10^3$ cell challenge with irradiated cell doses of up to $10^7$.

**Protective effects of pre-treatment with *C. parvum***

Groups of 10 mice received 0.2 ml of *C. parvum* i.v. or i.p. 7 days before challenge with tumour cells. When *C. parvum* is administered 7 days before injection of sheep red cells it produces a marked adjuvant effect (Scott, unpublished results).

*RI leukaemia.*—Challenge doses of both 10 and 100 cells were assayed (Fig 1A, B). In all cases there was a definite protective effect from *C. parvum* which was highly significant at the lower challenge dose and with little difference between the i.v. and i.p. routes of administration. It was noticeable that prolongation of survival of animals incompletely protected by *C. parvum* was accompanied by greater ascites development. Very little protection from *C. parvum* was
Fig. 2 A–C.—The effect of C. parvum pre-treatment on Hepatoma 129 in CBA (a, b) and BALB/c (c) mice. 1-4 mg C. parvum 7 days before challenge with 10 or 100 tumour cells. C. parvum i.v. (---), C. parvum i.p. (-- -- --), none (----).

D–E.—The effect of combined C. parvum pre-treatment and immunization on Hepatoma 129 cells in BALB/c mice. 1-4 mg C. parvum 7 days before immunization i.p. with 5.10^4 (d), 5.10^5 (e) irradiated tumour cells. Untreated mice (-----), immunization alone (+++). C. parvum i.v. + immunization (-----), C. parvum i.p. + immunization (-- -- --).

Fig. 3.—The effect of C. parvum pre-treatment on CBA-T3 fibrosarcoma in CBA mice. 1-4 mg C. parvum 7 days before challenge with either 10^3 or 10^4 tumour cells s.c. (top row) or i.p. (bottom row). C. parvum i.v. (-----), C. parvum i.p. (-- -- --), none (-----).
afforded against the poorly immunogenic RI-var, as compared with the original RI (Fig. 1C).

_Hepatom_ 129.—Challenges of both 10 and 100 cells in the CBA system and 100 cells in the BALB/c system were assayed (Fig. 2A–C). In CBA mice the protective effect was small, with little difference between _C. parvum_ i.v. and i.p. The situation was quite different with BALB/c mice: whereas _C. parvum_ i.v. had little effect, there was a strong protective effect following i.p. administration.

_CBAT-3 fibrosarcoma._—Both s.c. and i.p. challenge with 10^3 and 10^4 cells were investigated (Fig. 3): s.c. challenge with 10^3 cells proved too small for analysable results, although _C. parvum_ pre-treatment both i.p. and i.v. increased the number of survivors. At the 10^4 dose there was a definite effect with _C. parvum_ i.p. but very little with i.v. injection. With i.p. challenge of 10^3 cells both i.v. and i.p. _C. parvum_ afforded strong protection. This protection was maintained by i.p. _C. parvum_ against a 10^4 cell challenge, but the i.v. route was again less effective.

_L5178 leukaemia._—The effect of _C. parvum_ pre-treatment against a 100 cell challenge was minimal (Fig. 4).

_PC6 plasmacytoma._—Pre-treatment again afforded only slight protection against challenges of 10^3 and 10^4 cells (Fig. 4).

_Combined pre-treatment with _C. parvum_ and irradiated cells_

_C. parvum_ was injected 7 days before immunization with irradiated cells and the animals were challenged after a further 7 days. Groups of 10 mice were used.

_RI leukaemia._—The results of combining immunization by 5 x 10^4 or 5 x 10^5 irradiated cells with i.v. _C. parvum_ are shown in Fig. 1D–F. _C. parvum_ pre-treatment depressed the protective effect of immunization, more especially with the lower immunizing dose, but exerted its normal protective effect when given alone.

_Hepatom_ 129.—The outcome of combining immunization with _C. parvum_ pre-treatment was investigated in the BALB/c system. Two immunizing doses
TABLE I.—The Protective Effect of C. parvum Pre-treatment Against Experimental Mouse Tumours of Varying Immunogenicity

| Tumour and strain of origin | Tumour type          | Experimental host and challenge route | Immunogenicity* | C. parvum protection† |
|-----------------------------|----------------------|---------------------------------------|-----------------|-----------------------|
| RI                          | Ascites leukaemia    | CBA                                   | × ×             | ++ + +                |
| CBA                         | Fibrosarcoma         | i.p                                   | ≤ ×             | ± ± ± ±               |
| CBA-RI                      | Fibrosarcoma         | CBA                                   | × × ×           | + + + +               |
| H129                        | Ascites hepatoma     | i.p                                   | × × ×           | + + + +               |
| C3H                         | Ascites hepatoma     | BALB/e                                | × × ×           | + + + +               |
| L5178                       | Ascites leukaemia    | (BALB/c x DBA/2)F1                    | ×               | ± ± ± ±               |
| DBA/2                       | Asestes plasmacytoma | i.p                                   | ×               | ± ± ± ±               |
| BALB/e                      | Asestes plasmacytoma | i.p                                   | ×               | ± ± ± ±               |
| CBAT-3                      | Fibrosarcoma         | CBA                                   | ×               | + + + +               |
| CBA                         | Fibrosarcoma         | s.c                                   | ×               | + + + +               |
| CBA                         | Fibrosarcoma         | i.p                                   | ×               | + + + +               |

* Graded according to dose of irradiated tumour cells giving 50% protection against a challenge of 10–20 times the LD50 of living tumour cells: × × × 10^6–10^7; × × 10^5–10^6; × 10^4–10^5.
† Subjective grading from good (+ + +) to poor (±) protection.

(5 × 10^4 and 5 × 10^5) were used with both i.v. and i.p. C. parvum (Fig. 2D–E). Good protection was induced with i.p. C. parvum alone and such treatment did not modify the protective effect of immunization. Administration of C. parvum i.v., which alone afforded minimal protection, completely abolished the protective effect of the lower immunizing dose of irradiated cells and slightly reduced that of the high dose.

DISCUSSION

C. parvum pre-treatment was found to confer some degree of protection against a variety of highly adapted and rapidly growing mouse tumours, in agreement with reports by previous workers (Halpern et al., 1966; Woodruff and Boak, 1966; Lamansans et al., 1968).

It seemed likely that C. parvum would be more effective against the more immunogenic tumours and such a correlation is seen in the comparison of the normal and variant RI strains. The variation in results obtained with the different tumour systems serves to emphasize the complexity of the mechanisms involved, but the more striking effects were found in the immunologically reactive situations (Table I). C. parvum causes intense reticulo-endothelial stimulation (Halpern et al., 1964) and stimulated macrophages have been reported to become non-specifically cytotoxic to tumour cells (Alexander and Evans, 1971; Hibbs, Lambert and Remington, 1972). Such effects may explain, for example, the slight protection against the very poorly immunogenic PC6 tumour, but the suggested correlation between immunogenicity and C. parvum protection implies involvement of specific immunological defences in addition to any non-specific activation.

The i.p. route for C. parvum was somewhat more effective than the i.v. although both routes were similar with respect to spleen and liver enlargement and neither led to an increased peritoneal cell population at the time of challenge (authors' unpublished observations). That the difference cannot be explained simply by local i.p. effects is also shown by the greater efficacy of i.p. C. parvum in the s.c. CBAT-3 system.

In view of the known adjuvant activity of C. parvum, it was anticipated that combination with immunization would result in increased protection compared with the latter alone. However, in both the systems investigated, i.v.
C. parvum, given before immunization, decreased the protective effect, although i.p. C. parvum in the H129 system did not influence the immune response. It seems that while C. parvum treatment stimulates the defenses of the host against many tumors it may also, at least when given i.v., concomitantly depress some part of the immune response. Preliminary experiments both in vitro and in vivo have so far failed to reveal evidence for production of enhanced antibody and the fact that the depression can be overridden by larger immunizing doses also argues against such a mechanism. Enhanced growth of allogeneic tumour cells following immunization in conjunction with either complete or incomplete Freund's adjuvant has recently been reported (Zola, 1972), and again no evidence of enhancing antibody could be found, the effect being attributed to depression of cell-mediated cytotoxicity. Evidence of depressed cell-mediated (T lymphocyte) responses following C. parvum treatment comes from the demonstration of reduced delayed hypersensitivity (Asherson and Allwood, 1971), PHA responsiveness and GVH reactivity of lymphocytes (Scott, 1972).

Schedules for the immunotherapeutic treatment of human tumours have often included adjuvants, and C. parvum has begun to figure among these (Mathé, 1971). The present finding that this agent, at least under certain experimental conditions, can show an immunosuppressive component in its action urges caution in this field.

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