

FURTHER OBSERVATIONS ON THE EFFECT OF *C. PARVUM* AND ANTI-TUMOUR GLOBULIN ON SYNGENEICALLY TRANSPLANTED MOUSE TUMOURS

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Received for publication December, 1971

Summary.—The inhibitory effect of an i.v. or i.p. injection of *C. parvum* on intrastain transplants of a mammary carcinoma in A/HeJ mice has been confirmed, and it has been shown further that *C. parvum* inhibits the growth of transplants of sarcomata induced with methylcholanthrene both in this strain (members of which lack the fifth component of complement) and in CBA mice (which are not complement deficient). In experiments with the mammary carcinoma, 2 injections of *C. parvum* on days + 3 and + 9 were more effective than a single injection on day + 3; injections on days + 3 and + 6, or + 3 and + 12, appeared to be marginally less effective than on days + 3 and + 9, but the difference was not statistically significant.

Development of the CBA sarcoma was inhibited to about the same extent if, instead of treating the mouse with *C. parvum*, the tumour cells were pre-incubated with anti-tumour globulin (ATG) in the absence of complement prior to inoculation, and the effect of combining these procedures was much greater than that of either alone. Pre-incubation with ATG had a similar but less marked effect on the development of the mammary carcinoma but had no effect on the A/HeJ sarcoma. Injection (i.v.) of ATG did not inhibit the growth of any of the tumours in these experiments and possible reasons for this are discussed.

As already reported, the growth of intrastain transplants of a mammary carcinoma in A-strain mice may be inhibited to a moderate extent by treating the recipient with a single intravenous or intraperitoneal injection of an appropriate strain of *C. parvum* (Woodruff and Boak, 1966; Smith and Woodruff, 1968; Woodruff and Inchley, 1971*a*) or by incubating the tumour cells prior to inoculation with heterospecific antitumour globulin (ATG) (Woodruff and Smith, 1970). Significantly greater inhibition may be obtained by combining these procedures (Woodruff and Inchley, 1971*a*).

The experiments now reported extend this work in two directions. In the first place we have studied the effect of treating the animal with ATG, either alone or in combination with *C. parvum*. Secondly, in order to determine whether or not the results obtained previously were peculiar to the particular model chosen, in which the tumour was of viral

origin and the mice lacked the fifth component of complement (C5), we have set up similar experiments with 2 fibrosarcomata induced with methylcholanthrene, one in A/HeJ mice (*i.e.* the strain used in previous work with the mammary carcinoma) and one in CBA mice, which possess all components of the complement system.

MATERIALS AND METHODS

Six experiments were performed, 2 with A/HeJ mammary carcinomata, 2 with a fibrosarcoma induced in A/HeJ mice with methylcholanthrene and 2 with a fibrosarcoma induced in CBA mice by the same carcinogen. The detailed protocols are shown in Tables I, II and III. It should be noted that in these experiments *C. parvum* was injected on day + 3 (*i.e.* 3 days after tumour inoculation) and not on day - 2 as previously.

Mice.—The mice were adult (18–26 g) females of strain A/HeJ or CBA/H. The A/HeJ mice were obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine,

TABLE I.—*Experiments with Mammary Carcinoma Transplants in A/HeJ Mice*

Expt* Group	Tumour cells		Treatment of recipient		Mean tumour diameter on day +21		Mean tumour diameter on day +43	
	No. of mice* or untreated†	Dose	<i>C. parvum</i>	ATG	Observed values (mm)	Group mean (mm)	Observed values (mm)	Group mean (mm)
1	6	10 ⁶	Nil	Nil	7, 5, 1, 1, 3, 0	2.8	18, 13, 10, 14, 16, 6	12.8
2	5	10 ⁶	Nil	Nil	2, 2, 0, 3, 0	1.1	14, 10, 9, 8, 0	8.2
3	6	10 ⁴	Nil	Nil	0, 0, 0, 0, 0	0.0	0, 12, 0, 0, 0, 0	2.0
4	5	10 ⁶	0.2 ml i.p. day +3	Nil	1, 1, 0, 0, 1	0.6	5, 6, 1, 1, 4	3.4
5	6†	10 ⁶	Nil	5 mg i.v. days +1, +2	4, 5, 5, 0, 2, 0	2.7	20, 19, 13, 17, 6	15.0
6	6	10 ⁶	0.2 ml i.p. day +3	5 mg i.v. days +1, +2	1, 1, 2, 3, 0, 5	2.0	17, 8, 13, 13, 6, 12	11.5
7	5	Pre-inc. ATG	Nil	Nil	2, 1, 0, 0, 2, 1	1.0	11, 10, 0, 0, 11, 6	6.3
8	6	Pre-inc. ATG	0.2 ml i.p. day +3	Nil	1, 0, 0, 0, 0, 0	0.2	7, 4, 1, 6, 4, 5	4.5
9	6	Pre-inc. NRG	Nil	Nil	0, 1, 4, 5, 4, 1	2.5	12, 10, 15, 19, 14, 11	13.5
10	5	Pre-inc. NRG	0.2 ml i.p. day +3	Nil	0, 0, 0, 1, 2	0.6	7, 6, 9, 9, 9	8.0
First	1	8	Untreated	Nil	14, 9, 12, 13, 15, 10, 14, 6	11.6		
Second	2	8	Untreated	Nil	12, 12, 10, 9, 3, 9, 12, 8	9.4		
	3	8	Untreated	Nil	3, 11, 10, 9, 5, 8, 6, 10	7.8		
	4	8	Untreated	Nil	6, 6, 5, 5, 9, 6, 5, 5	5.9		
	5	8	Untreated	Nil	6, 5, 8, 10, 3, 14, 1, 11	7.2		

* Different tumours were used in these 2 experiments. Both had been transplanted once previously. There were 6 mice in each group in the first experiment and 8 in each group in the second. The number of mice tabulated is the number alive 14 days after tumour inoculation.

† Pre-incubation was performed by incubating 2.10⁷ cells in 2.6 ml Dulbecco's solution containing 6.5 mg of the corresponding anti-tumour globulin (ATG) or normal rabbit IgG (NRG) for 60 min at 37°C without added complement.

‡ One animal died after 24 days.

U.S.A.; the CBA mice were bred in our laboratory from a strain obtained originally from the MRC Laboratory Animals Centre, Carshalton, Surrey.

Tumours.—The mammary carcinomata originated spontaneously in old female A/HeJ mice. The sarcomata were induced by giving a single intramuscular injection of 0.5 mg methylcholanthrene in 0.1 ml trioctanoin to A/HeJ or CBA female mice aged 8–10 weeks. The tumours were propagated routinely by subcutaneous (s.c.) transplantation of a small piece of tissue, but in the experiments transplantation was performed by s.c. injection of cell suspensions prepared with pronase as described previously (Woodruff and Boak, 1966). The proportion of viable cells, determined by a dye-exclusion test with trypan blue, ranged from 80 to 90%. The tumour dose is expressed as the number of viable cells.

The 2 mammary carcinomata had both been transplanted once, before being used in the experiment. Both sarcomata had been

transplanted 4 times before being used for the first time experimentally.

C. parvum.—A formalin-killed suspension, Batch EZ174, obtained from the Wellcome Foundation by courtesy of Dr J. Cameron, was used throughout. This is the same as the first of the 2 preparations used in our last experiment (Woodruff and Inchley, 1971a).

ATG.—ATG (A/HeJ–Ca) was prepared by salt precipitation and batch chromatography from the serum of rabbits immunized with a mixture of tumour cells prepared with pronase from several different A/HeJ mammary tumours according to the schedule described previously (Woodruff and Inchley, 1971a).

ATG (A/HeJ–Sa) and ATG (CBA–Sa) were prepared similarly from the serum of rabbits immunized in the same manner with cells prepared with pronase from transplants of one or other of the fibrosarcomata used in the experiments. The cytotoxic titres⁻¹ of these preparations by the techniques previously described

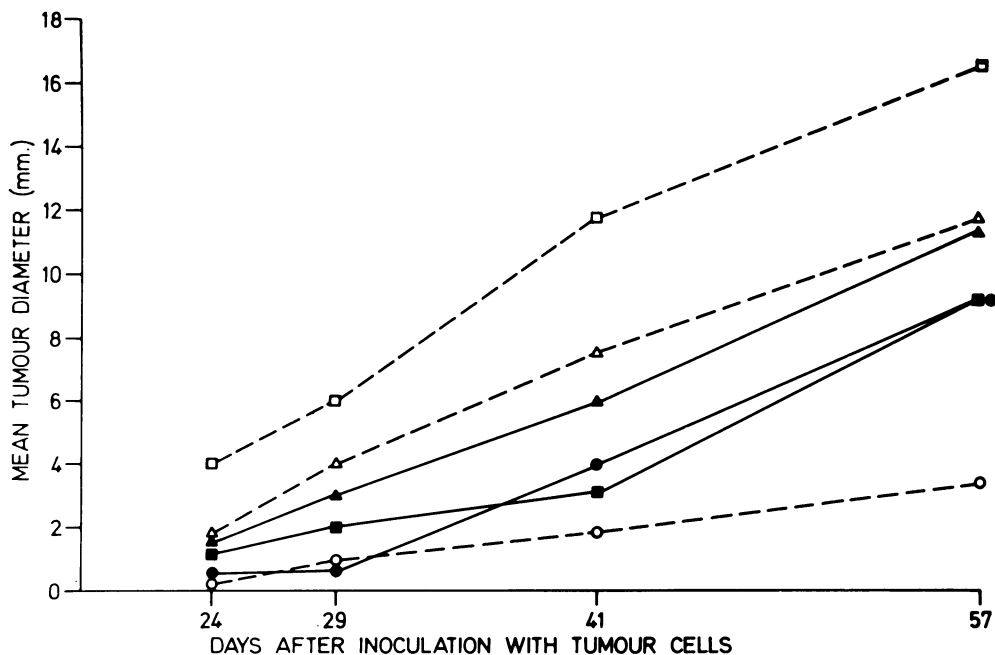


FIG. 1.—Effect of initial cell dose, pre-incubation of tumour cells with ATG and injection of *C. parvum*, on the growth of mammary tumour transplants in A/HeJ mice. First experiment. □—□ 10⁶ viable tumour cells s.c. No treatment. △—△ 10⁵ viable tumour cells s.c. No treatment. ○—○ 10⁴ viable tumour cells s.c. No treatment. ■—■ 10⁶ viable tumour cells s.c. *C. parvum* i.p. day + 3. ▲—▲ 10⁶ viable tumour cells s.c. Cells were pre-incubated with ATG. ●—● 10⁶ viable tumour cells s.c. Cells were pre-incubated with ATG. *C. parvum* i.p. day + 3.

TABLE II.—*Experiments with Fibrosarcoma Transplants in A/HeJ Mice*

Expt* Group	No. of mice* Pre-incubated† or untreated	Tumour cells		Treatment of recipient		Mean tumour diameter on day +22		No. of mice which eventually developed tumours	
		Dose	<i>C. parvum</i>	<i>C. parvum</i>	ATG	Observed values (mm)	Group mean (mm)		
First	1	9	Untreated	10 ⁴	Nil	Nil	16, 17, 17, 16, 17, 16, 14, 18	16.3	9
	2	6	Untreated	10 ³	Nil	Nil	15, 14, 13, 12, 15, 14	13.8	6
	3	6	Untreated	10 ²	Nil	Nil	11, 13, 14, 13, 11, 13	12.5	6
	4	6	Untreated	10 ⁴	0.2 ml i.p. day +3	Nil	12, 11, 1, 11, 2, 4	6.8	6
	5	6	Untreated	10 ⁴	Nil	5 mg i.v. days +1, +2	17, 17, 17, 16, 17, 16	16.7	6
	6	6	Untreated	10 ⁴	0.2 ml i.p. day +3	5 mg i.v. days +1, +2	10, 12, 13, 11, 12, 9	11.2	6
	7	6	Pre-inc. ATG	10 ⁴	Nil	Nil	16, 15, 17, 16, 15, 16	15.8	6
	8	6	Pre-inc. ATG	10 ⁴	0.2 ml i.p. day +3	Nil	11, 9, 11, 11, 9, 9	10.0	6
	9	6	Pre-inc. NRG	10 ⁴	Nil	Nil	17, 17, 14, 17, 15, 18	16.3	6
	10	6	Pre-inc. NRG	10 ⁴	0.2 ml i.p. day +3	Nil	9, 12, 4, 11, 10, 9	9.2	6
Second	1	8	Untreated	10 ⁴	Nil	Nil	2, 7, 9, 9, 7, 6, 5, 2	5.9	8
	2	8	Untreated	10 ⁴	0.2 ml i.p. day +3	Nil	0, 3, 4, 0, 2, 0, 3, 0	1.5	6
	3	8	Untreated	10 ⁴	0.2 ml i.v.	Nil	0, 0, 0, 0, 1, 0, 0, 0	0.1	6
	4	8	Untreated	10 ³	Nil	Nil	6, 6, 3, 6, 6, 3, 0, 3	4.1	7
	5	8	Untreated	10 ³	0.2 ml i.p. day +3	Nil	0, 0, 0, 0, 1, 0, 0, 0	0.1	2
	6	8	Untreated	10 ²	Nil	Nil	0, 0, 1, 0, 0, 2, 3, 0	0.8	1
	7	8	Untreated	10 ²	0.2 ml i.p. day +3	Nil	0.0.0.0.0.0.0.0	0.0	0

* Successive transplant generations of the same tumour were used in these experiments. The tumour had been transplanted 4 times prior to the first experiment. The number of mice tabulated is the number set up in each group. There were no early deaths in these 2 experiments.
 † Pre-incubation was performed by incubating 2.10⁷ cells in 2.6 ml Dulbecco's solution containing 6.5 mg of the corresponding anti-tumour globulin (ATG) or normal rabbit IgG (NRG) for 60 min at 37°C without added complement.

(Woodruff and Smith, 1970), using guinea-pig C and taking 50% cell death as the end point, were 64, 32 and 64 respectively. Normal rabbit IgG (NRG) was prepared from the pooled serum of normal rabbits.

Pre-incubation of tumour cells.— $2 \cdot 10^7$ cells were incubated in 2.6 ml Dulbecco's solution containing 6.5 mg of the corresponding ATG for 60 min at 37°C without added complement.

RESULTS

The results are shown in the tables and figures. They may be summarized as follows:

A/HeJ mammary carcinoma

In the first experiment (Table I; Fig. 1) a single i.p. injection of *C. parvum* on day +3 appeared to be at least as effective in inhibiting tumour growth as an injection on day -2 had been in the

previously reported experiments. Pre-incubation of tumour cells with ATG, though not with NRG, resulted in moderate inhibition of tumour growth. Combination of both these procedures caused marked inhibition initially but after about 5 weeks the mean tumour diameter in this group of mice did not differ significantly from that of *C. parvum* treated mice which received non-incubated cells. Intravenous injection of ATG alone had no demonstrable effect in this experiment, and if anything it appeared to reduce the inhibitory effect of a subsequent i.p. injection of *C. parvum*.

In the second experiment (Table I) the tumour grew unusually quickly in the control mice and the inhibitory effect of a single i.p. injection of *C. parvum* was correspondingly small; 2 doses, however, had quite a marked effect. Administra-

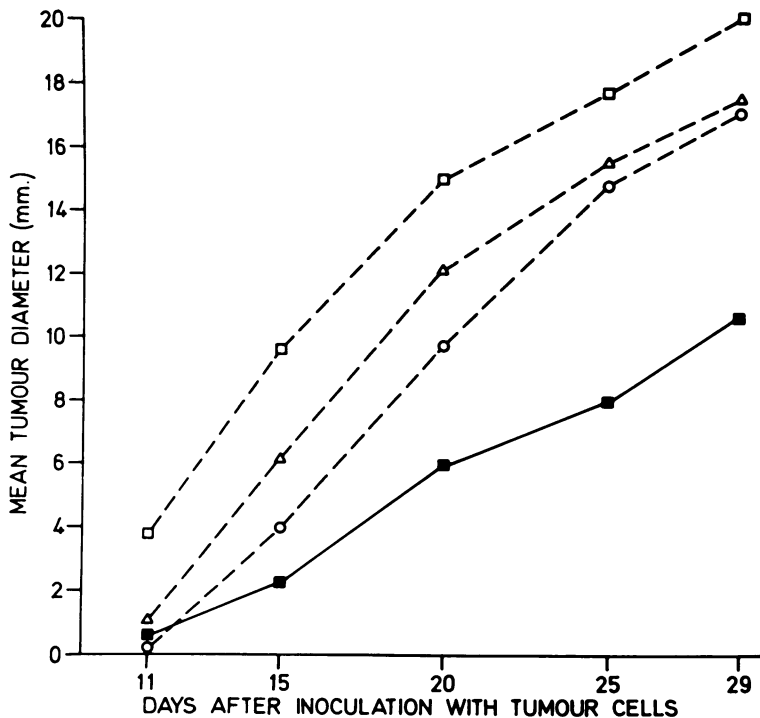


FIG. 2.—Effect of initial cell dose and injection of *C. parvum* on the growth of sarcoma transplants in A/HeJ mice. First experiment. □----□ 10^4 viable tumour cells s.c. No treatment. △----△ 10^3 viable tumour cells s.c. No treatment. ○----○ 10^2 viable tumour cells s.c. No treatment. ■——■ 10^4 viable tumour cells s.c. *C. parvum* i.p. day + 3.

tion of the second dose on day + 9 appeared to be marginally more effective than on day + 6 or + 12 but the difference is not statistically significant.

A/HeJ sarcoma

In the first experiment (Table II; Fig. 2) the tumour grew quite rapidly even after inoculation of only 100 viable cells. A single i.p. injection of *C. parvum* on day + 3 caused marked inhibition of growth. Neither pre-incubation of the tumour cells with ATG nor i.v. injection of ATG alone had any demonstrable effect, nor did these procedures augment the effect of a subsequent injection of *C. parvum*; indeed i.v. injection of ATG appeared if anything to reduce this.

The second experiment (Table II; Fig. 3) provided further evidence of the powerful inhibitory effect of a single

injection of *C. parvum*, and the results suggest that intravenous injection is even more effective than intraperitoneal.

CBA/H sarcoma

In the first experiment (Table III; Fig. 4) growth of the tumour was inhibited to about the same extent by pre-incubation of the cells with ATG or by a single i.p. injection of *C. parvum*. Pre-incubation of the tumour cells with ATG combined with injection of *C. parvum* was still more effective. Intravenous injection of *C. parvum* seemed initially to be more effective than i.p. injection but after a few weeks the difference was no longer apparent.

In the second experiment (Table III; Fig. 5) the inhibitory effect of *C. parvum* was confirmed, but i.p. injection was no less effective than i.v. As in the experi-

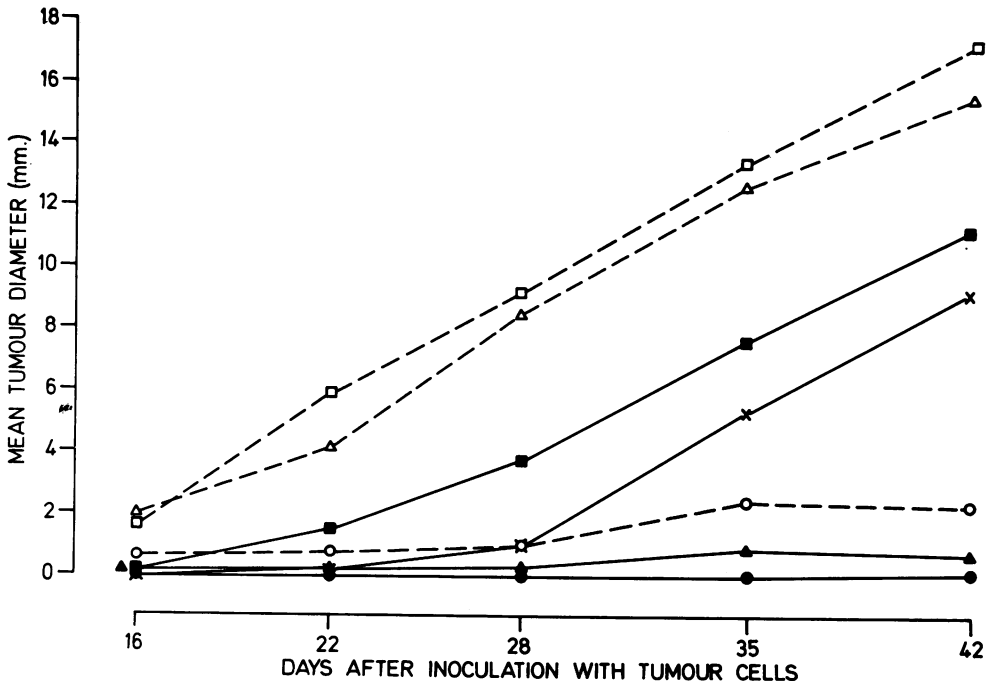


FIG. 3.—Effect of initial cell dose and injection of *C. parvum* on the growth of sarcoma transplants in A/HeJ mice. Second experiment. □---□ 10⁴ viable tumour cells s.c. No treatment. ■---■ 10⁴ viable tumour cells s.c. *C. parvum* i.p. day + 3. ×---× 10⁴ viable tumour cells s.c. *C. parvum* i.v. day + 3. △---△ 10³ viable tumour cells s.c. No treatment. ▲---▲ 10³ viable tumour cells s.c. *C. parvum* i.p. day + 3. ○---○ 10² viable tumour cells s.c. No treatment. ●---● 10² viable tumour cells s.c. *C. parvum* i.p. day + 3.

TABLE III.—*Experiments with Fibrosarcoma Transplants in CBA Mice*

Expt	Group	Tumour cells		Treatment of recipient		Mean tumour diameter		Group Mean (mm)
		No. of mice* or untreated	Dose	<i>C. parvum</i>	ATG	Observed values (mm)	Group mean (mm)	
First	1	8	10 ⁴	Nil	Nil	3, 3, 3, 4, 7, 3, 2, 3	3.5	17, 14, 18, 19, 18, 18, 14, 19
	2	8	10 ⁴	0.2 ml i.p. day +3	Nil	0, 0, 2, 2, 1, 1, 4, 2	1.5	12, 10, 17, 10, 0, 9, 16, 10
	3	6	10 ⁴	0.2 ml i.v. day +3	Nil	0, 1, 0, 0, 2, 1	0.7	11, 13, 12, 11, 12, 8
	4	8	10 ⁴	Pre-inc. ATG	Nil	2, 1, 4, 2, 2, 1, 1, 1	1.8	12, 15, 13, 9, 12, 7, 7, 1
	5	8	10 ⁴	Pre-inc. ATG	Nil	0, 0, 0, 0, 0, 0, 2	0.3	1, 0, 3, 0, 0, 10, 0, 9
	6	8	10 ³	Nil	Nil	1, 4, 2, 3, 3, 1, 2,	2.4	11, 16, 7, 14, 15, 19, 15, 18
	7	7	10 ³	0.2 ml i.v. day +3	Nil	0, 1, 4, 0, 2, 0, 1	1.0	1, 10, 13, 8, 4, 7, 10
	8	8	10 ²	Nil	Nil	3, 1, 2, 3, 0, 1, 3, 4	2.1	12, 1, 16, 16, 13, 7, 15, 16
	9	8	10 ²	0.2 ml i.v. day +3	Nil	1, 1, 1, 1, 0, 2, 1, 2	1.1	7, 11, 9, 8, 7, 0, 8, 7
Second	1	8	10 ⁴	Nil	Nil	2, 3, 4, 4, 4, 3, 2, 4	3.3	16, 20, 20, 20, 19, 20, 20, 15
	2	7	10 ⁴	0.2 ml i.p. day +3	Nil	0, 0, 1, 3, 2, 0, 0	0.9	12, 13, 7, 15, 15, 12, 7
	3	6	10 ⁴	Nil	5 mg i.v. days +1, +2	5, 5, 3, 4, 1, 5	3.8	20, 20, 20, 20, 12, 20
	4	5	10 ⁴	0.2 ml i.p. day +3	5 mg i.v. days +1, +2	0, 0, 1, 1, 1	0.6	13, 13, 19, 16, 12
	5	6	10 ⁴	0.2 ml i.v. day +3	5 mg i.v. days +1, +2	1, 2, 3, 1, 0, 3	1.7	15, 15, 20, 11, 12, 19

* There were initially 8 mice in each group. The number shown is the number alive 14 days after tumour inoculation.

ments with the A/HeJ sarcoma, however, injection of ATG did not inhibit growth of the tumour and appeared to reduce the inhibitory effect of a subsequent dose of *C. parvum*.

DISCUSSION

It seems clear that the inhibitory effect of *C. parvum* is not peculiar to the model previously investigated but extends to intra-strain transplants of chemically induced sarcomata in both complement (C5) deficient and non-complement deficient mice. The effect of *C. parvum* on cholanthrene-induced sarcomata in CBA mice has also been studied by Currie and Bagshawe (1970). The inhibition of tumour growth in animals treated with *C. parvum* alone appears to have been weak in comparison with that obtained in the present experiment, probably because of differen-

ces in the strain of organism and in the method of preparing the vaccine, which was heat-killed (as against formalin-killed). It is noteworthy, however, that despite this, Currie and Bagshawe observed a marked synergistic effect when a single injection of *C. parvum* was preceded by a course of injections of cyclophosphamide.

The effect of pre-incubating the tumour cells in the present experiments with heterospecific ATG was variable, inhibition of growth being greatest with the CBA sarcoma but clearly demonstrable also with the A-strain mammary carcinoma. The synergistic effect of combining pre-incubation of the cells and injection of *C. parvum*, which was so marked in previous experiments with the A-strain mammary carcinoma, was less striking in the present experiment with this tumour, possibly because of the

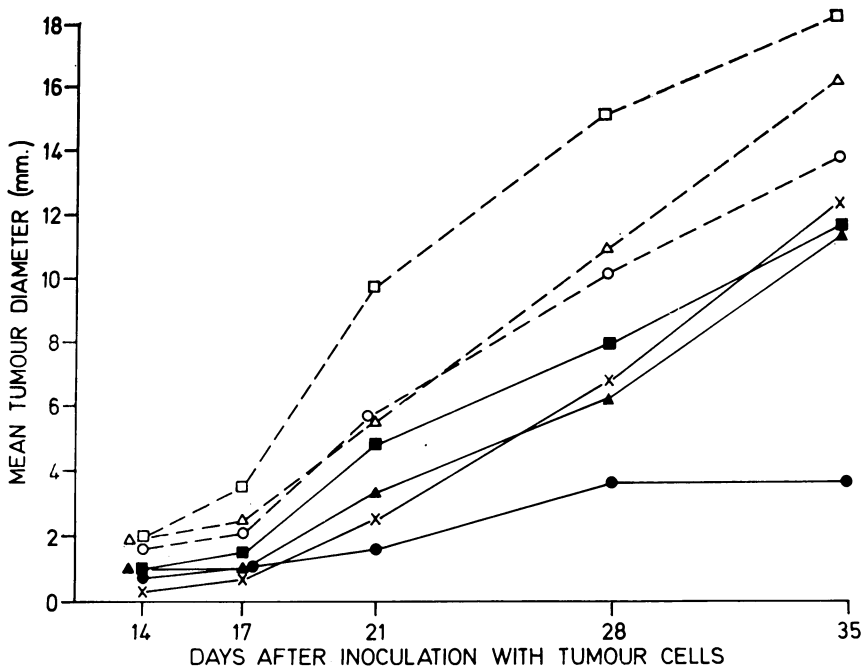


FIG. 4.—Effect of initial cell dose, pre-incubation of tumour cells with ATG, and injection of *C. parvum*, on the growth of sarcoma transplants in CBA/H mice. First experiment. □----□ 10⁴ viable tumour cells s.c. No treatment. △----△ 10³ viable tumour cells s.c. No treatment. ○----○ 10² viable tumour cells s.c. No treatment. ■——■ 10⁴ viable tumour cells s.c. *C. parvum* i.p. day + 3. ×——× 10⁴ viable tumour cells s.c. *C. parvum* i.v. day + 3. ▲——▲ 10⁴ viable tumour cells s.c. Cells were pre-incubated with ATG. ●——● 10⁴ viable tumour cells s.c. Cells were pre-incubated with ATG. *C. parvum* i.p. day + 3.

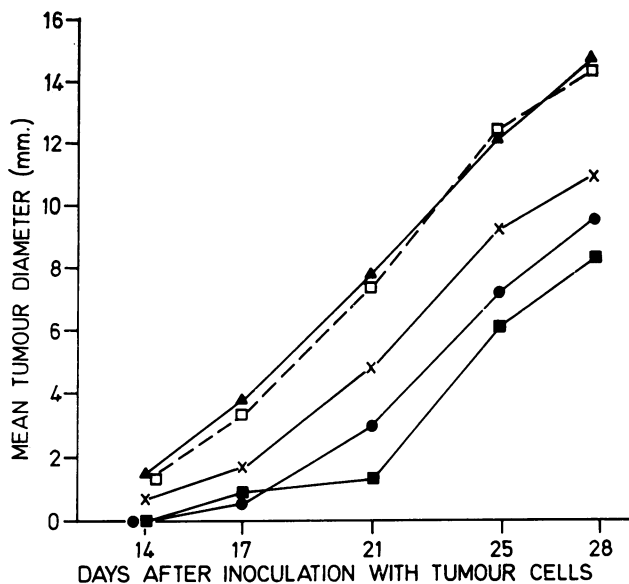


FIG. 5.—Effect of injection of *C. parvum* and ATG on the growth of sarcoma transplants in CBA/H mice. Second experiment. All the mice received 10^4 viable cells s.c. □—□ No treatment. ■—■ *C. parvum* i.p. day + 3. ▲—▲ ATG 5 mg i.v. day + 1, + 2. ●—● ATG 5 mg i.v. day + 1, + 2. ×—× ATG 5 mg i.v. day + 1, + 2, *C. parvum* i.p. day + 3.

different timing of the *C. parvum* injection, but was quite dramatic with the CBA/H sarcoma.

The observation that i.v. injection of ATG did not inhibit the growth of any of the tumours studied is in line with the experience of other investigators (see Motta, 1971, for review), who have found that, with a few exceptions, treatment with antiserum is ineffective with mouse tumours other than leukoses.

In discussing possible reasons for the failure of passive immunotherapy it is convenient to begin by considering the factors which determine the susceptibility of cells to destruction consequent on exposure to antibody and complement. These include intrinsic properties of the cells, the specificity and class of antibody, the availability of necessary complement components, and the milieu in which the reaction occurs.

In the case of lysis of both normal and neoplastic nucleated cells by isoantibody *in vitro*, the concentration and distribution of corresponding antigenic determin-

ants on the surface of the cell are of crucial importance (Möller and Möller, 1962; Winn, 1965), though other properties of the cell, such as the capacity to repair lesions resulting from activation of complement on the cell surface, may also be relevant. Lysis of nucleated cells by heterospecific antibody *in vitro* has not been extensively analysed, but it might be expected that the number of combining sites would be greater than with isoantibodies and in consequence less likely to be a limiting factor, and results obtained in this laboratory (Woodruff and Inchley, 1971*b*) with one particular system are consistent with this suggestion. Whether or not this holds good generally, however, it seems clear that the failure of passive immunization in the present experiments cannot be attributed wholly to some intrinsic property of either the tumour cells or the antibody, since, when sensitized with the corresponding antibody, the cells of all the tumours were lysed *in vitro* by guinea-pig complement, and, as mentioned above, those of two of them

proved to be at a disadvantage as compared with normal cells when injected *in vivo*.

It seems likely that the explanation is to be found in the amount of antibody reaching the tumour cells under the conditions of the experiments, and on the availability of complement. To investigate this we are studying the uptake of radioactively labelled antibody by tumours *in vivo* and the complementary activity of serum from tumour bearing animals. Another approach is to try to prepare antibody which is more highly tumour specific, and therefore less liable to be mopped up by normal tissues, and to study the effect of injecting this alone or with exogenous complement.

Another possibility, which may also be tested by using sera which are more tumour specific, is that the ATG used in the present experiments exerted an immunosuppressive effect which was sufficient to counterbalance any damaging effect it had on the tumour.

Various agents which stimulate immunological responsiveness in a non-specific way, including Bacillus Calmette Guérin (BCG) and synthetic polynucleotides, have been tried clinically in patients with acute lymphoblastic leukaemia by Mathé (Mathé *et al.*, 1968; Mathé *et al.*, 1970; Mathé, 1970) and some of the results have been encouraging. In our hands (Woodruff and Dunbar, unpublished) these have proved much less effective against mouse tumours than the preparation of *C. parvum* used in the present experiments. There would seem therefore to be a good case for undertaking a preliminary clinical trial of this preparation, either alone or in combination with chemotherapy or some other form of immunotherapy, in selected patients with residual cancer, provided that the criteria of uniformity and safety applicable to bacterial vaccines can be met.

We gratefully acknowledge a generous gift of *C. parvum* from the Wellcome Foundation (Dr J. Cameron). We are indebted also to Mrs Evelyn Pawley for skilled technical assistance.

The work was supported by a grant from the Medical Research Council.

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