SYNERGISTIC INHIBITION OF MAMMARY CARCINOMA TRANSPLANTS IN A-STRAIN MICE BY ANTITUMOUR GLOBULIN AND *C. PARVUM*

M. F. A. WOODRUFF AND M. P. INCHLEY

From the Department of Surgery, University of Edinburgh

Received for publication July 27, 1971

SUMMARY.—It has been confirmed that the growth of intrastrain transplants of a mammary carcinoma in A/HeJ mice is inhibited to a moderate extent by giving the prospective recipient an intravenous injection of killed *C. parvum* 2 days before tumour inoculation. Intraperitoneal injection of *C. parvum* gave similar results but subcutaneous injection was less effective. Incubation of tumour cells with heterospecific antitumour globulin (ATG) in the absence of complement before inoculation sometimes but not always resulted in moderate inhibition of tumour growth. When pre-incubation of tumour cells with ATG and treatment of the host with *C. parvum* were combined the inhibitory effect was much greater than that produced by either procedure alone, and was roughly equivalent to reducing the dose of viable cells in control animals by a factor of 100. Since A/HeJ mice lack the fifth component of complement (C′5) it was expected that this serum would be ineffective as a source of C′ in *in vitro* cytotoxic tests but effective in opsonisation tests. This has been confirmed. The possible significance of this finding in relation to the synergistic effect of ATG and *C. parvum* is discussed.

As already reported (Woodruff and Smith, 1970), the growth of isogeneic mammary carcinoma transplants in A-strain mice may be inhibited by treating the recipient with heterospecific antitumour globulin (ATG), but the degree of inhibition is variable and never very marked. It was anticipated that pre-incubation of tumour cells with ATG in the absence of complement (C′) before inoculation would have a more decisive effect. As reported below, however, we have found that, while tumour growth may be inhibited to a modest extent following this procedure, it may, on the other hand, be unaffected or even facilitated.

It was reported by Cinader, Dubiski and Wardlaw (1964) that A-strain mice, including in particular mice of the substrain A/HeJ which we have used extensively, lack a component of the complement system which has been subsequently identified by Nilsson and Müller-Eberhard (1967) as C′5. This is consistent with our own previously unreported finding that, despite precautions to avoid inactivation, including collection over ice (Borsos and Cooper, 1961), A/HeJ serum (unlike that of CBA and C57Bl mice) is completely ineffective when used as a substitute for guinea pig C′ in titrating haemolytic and cytotoxic antibody, whereas it is effective in opsonisation tests. Such inhibition of tumour growth as occurs in consequence of treatment of either the tumour cells preceding inoculation or the recipient with ATG, therefore, cannot be attributed to C′-dependent lysis, and the most likely explanation of the observations cited would seem to be that ATG

functions as an opsonin, and that its effectiveness depends on the level of macrophage activity in the host.

If this hypothesis is correct, procedures which stimulate phagocytosis by macrophages might be expected to potentiate the tumour-inhibiting effect of ATG.

One such procedure is administration of a killed culture of *C. parvum* (Halpern, Prevot, Biozzi, Stiffel, Mouton, Morard, Bouthillier and Decreusefond, 1963). It has already been reported from this laboratory that intravenous injection of this material, either 2 days before or 8–12 days after subcutaneous inoculation of viable mammary carcinoma cells in A-strain mice, significantly delays growth of the tumour (Woodruff and Boak, 1966; Smith and Woodruff, 1968). The present experiments were therefore designed to test the prediction that treatment of the tumour cells with ATG and of the host with *C. parvum* would combine synergistically, so that the degree of tumour inhibition resulting from the two procedures together would exceed that expected from simple summation of their individual effects. As a preliminary we have tested two more strains of *C. parvum*, and compared the effect of different routes and schedules of immunization.

MATERIALS AND METHODS

Three experiments were performed. The protocols are shown in Tables I–III.

First experiment.—This was designed to assess the effect of a single subcutaneous (s.c.), intraperitoneal (i.p.) or intravenous (i.v.) injection of *C. parvum* on the growth of a subcutaneous intrastrain transplant of a mouse mammary carcinoma. The *C. parvum* was given 2 days before tumour transplantation. This particular time interval was chosen in the light of previously reported experiments with other strains of *C. parvum* (Woodruff and Boak, 1966; Smith and Woodruff, 1968), and a preliminary trial with the strains used in the present investigation, which showed that injection 7 days preceding tumour transplantation had relatively little effect.

Second experiment.—This was designed with two objects in mind. The first was to extend the scope of the previous experiment (a) by comparing the effect of two i.p. doses of *C. parvum*, given on Days −2 and +3 with a single dose on Day −2 (reckoning the day of tumour transplantation as Day 0), and (b) by studying the effect of a single dose of *C. parvum* 2 days before excision of an established tumour transplant and re-inoculation of a measured number of viable cells. The second object was to determine the effect of incubating tumour cells with ATG before injecting them into untreated mice or mice which had been given a single i.p. dose of *C. parvum* 2 days previously.

Third experiment.—The third experiment was designed to provide further information concerning the separate and combined effects of (a) incubating tumour cells with ATG before transplantation, and (b) treating the prospective recipient with *C. parvum.*

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

Tumour transplantation.—Transplantation was performed by s.c. injection of a tumour cell suspension prepared with pronase, as described by Woodruff and Boak (1966), from a second generation transplant of a mammary carcinoma which had arisen spontaneously in an old A/HeJ female. The proportion of viable cells determined by a dye-exclusion test with trypan blue, ranged from 75 to 90 per

cent. The tumour dose was expressed as the absolute number of viable tumour cells injected.

The animals were examined thrice weekly. When a tumour became palpable its mean diameter was determined with callipers.

C. parvum.—Formalin-killed cultures of two different strains of *C. parvum* were used. One preparation was obtained from the Wellcome Foundation (Batch EZ174) by courtesy of Dr. J. Cameron; the other (Reticulostimuline) from the Pasteur Institute, Paris, by courtesy of Professor Marcel Raynaud. We have designated these W2 and P2 respectively to distinguish them from other preparations obtained previously from the same two sources. The nitrogen content, by microKjeldahl analysis, was 0·96 mg./ml. for W2 and 1·18 mg./ml. for P2. Both preparations were given in a dosage of 0·2 ml. W2 was used in all three experiments; P2 in the third experiment only.

Preparation and use of ATG.—Antitumour serum (ATS) was prepared by immunizing rabbits with a mixture of tumour cells prepared with pronase from intrastrain transplants of several tumours of the type described above, according to the following schedule:

Day 0 100×10^6 cells in Freund's complete adjuvant distributed between the four footpads, and 400×10^6 cells in physiological saline i.p.
Day 28 500×10^6 cells in physiological saline i.p.
Day 35 500×10^6 cells in physiological saline i.p.

The rabbits were bled (50 ml.) from an ear vein on Day 45 and exsanguinated by cardiac puncture on Day 46. The blood was allowed to clot, and the serum was separated, pooled and inactivated by heating for 30 minutes at 56° C.

ATG was prepared from ATS by two precipitations with an equal volume of 32 per cent sodium sulphate, followed by batch chromatography on Whatman DE11 DEAE=cellulose using a pH 6·5 0·02 M phosphate buffer, as described by Anderson, James and Woodruff (1967). Immunoelectrophoresis revealed only traces of impurities with an electrophoretic mobility greater than the IgG.

Different batches of ATG were used in the second and third experiments, but they both had an *in vitro* cytotoxic titre^{-1} of 128.

Pre-incubation of tumour cells.—$2 \cdot 10^7$ cells were incubated in 2·6 ml. Dulbecco's solution containing 6·5 mg. ATG at 37° C. in a water bath. As a rule no C′ was included, but as an additional control in the third experiment one group of mice (group 7) were given cells which had been pre-incubated as described above except that 0·65 ml. undiluted guinea pig serum had been added as a source of C′.

RESULTS

The results are summarised in the tables and figures. As reported previously (Woodruff and Boak, 1966), after a lag period during which no tumour was palpable, there was typically a period of some weeks during which tumour diameters increased in an approximately linear manner with time. Where necessary we have compared the mean diameter of tumours in treated and control animals at a time when the latter were at or a little beyond the mid-point of this phase by a t-test, using either the standard test or, when the variances in the groups compared were significantly different, Bailey's (1959) modification. Sometimes however the difference in tumour growth in different groups was obvious on inspection and statistical analysis was not required.

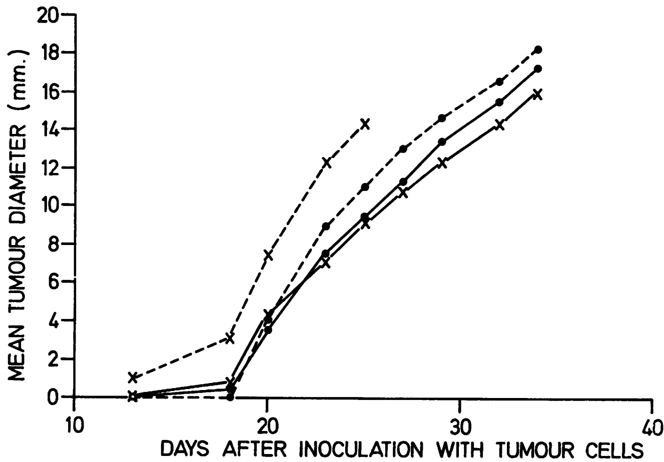

FIG. 1.—First experiment. Effect on tumour growth of a single i.v., i.p. or s.c. injection of *C. parvum*.

×⋯⋯⋯× No treatment.
×———× *C. parvum* i.v.
●———● *C. parvum* i.p.
●⋯⋯⋯● *C. parvum* s.c.

It seems clear from the first experiment (Table I; Fig. 1) that tumour growth was delayed by administration of *C. parvum* by any of the three routes tested, but intravenous injection was significantly more effective than subcutaneous. The difference between intraperitoneal and intravenous injection is not significant.

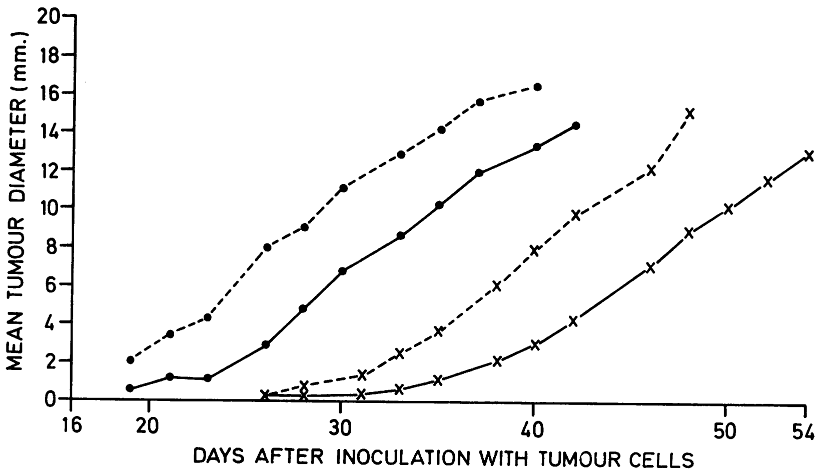

FIG. 2.—Second experiment. Effect of an i.p. injection of *C. parvum* on the growth of (1) a primary and (2) a secondary tumour transplant.

×⋯⋯⋯× Primary transplant. No treatment.
×———× Primary transplant. *C. parvum*.
●⋯⋯⋯● Secondary transplant. No treatment.
●———● Secondary transplant. *C. parvum*.

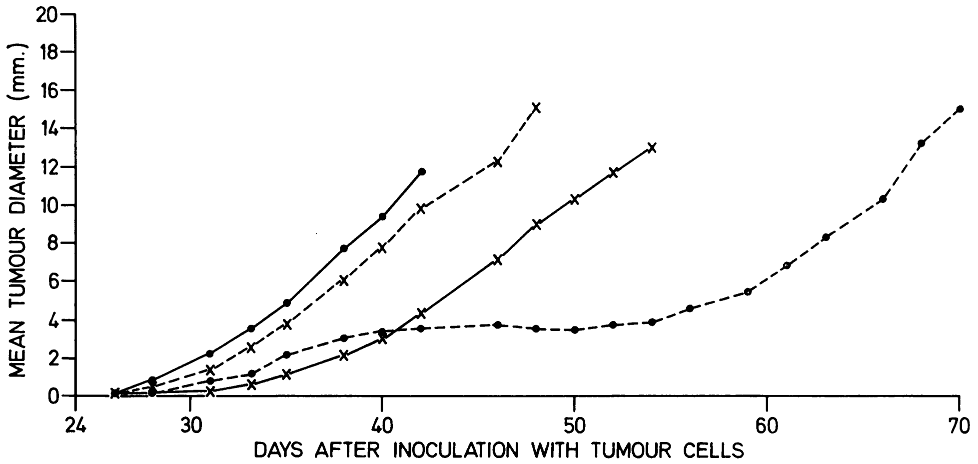

FIG. 3.—Second experiment. Separate and combined effects on tumour growth of (1) i.p. injection of the host with *C. parvum* (W2) and (2) pre-incubation of tumour cells with ATG (Batch E4Y1).

× ··········· × No treatment.
× ——— × *C. parvum*.
● ——— ● Pre-incubation with ATG.
● ··········· ● *C. parvum* and pre-incubation.

It seems possible, particularly in view of the large variance in tumour size in the mice given i.p. *C. parvum*, that a difference might be demonstrated by using more animals, but in the light of the findings it seemed reasonable to adopt the i.p. route in preference to the less convenient i.v. route throughout the rest of the investigation.

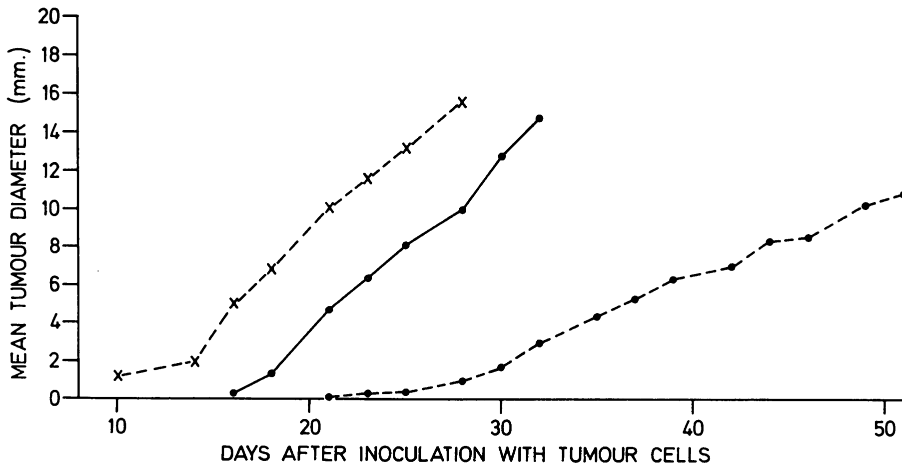

FIG. 4.—Third experiment. Influence of cell dose on tumour growth.

× ··········· × 10^6 cells.
● ——— ● 10^5 cells.
● ··········· ● 10^4 cells.

TABLE I.—*First Experiment—see also Fig.* 1

There were 7 mice in each group. All mice received 10^7 viable tumour cells by subcutaneous injection to R. flank on Day 0.

Group	Treatment	Mean tumour diameter on Day +25: Observed values mm.	Group mean mm.
1	Nil	13, 14, 16, 17, 10, 14, 15	14·1
2	*C. parvum* (W2) 0·2 ml. s.c. on Day −2	12, 11, 11, 11, 9, 12, 11	11·0
3	*C. parvum* (W2) 0·2 ml. i.p. on Day −2	9, 6, 13, 8, 9, 12, 10	9·6
4	*C. parvum* (W2) 0·2 ml. i.v. on Day −2	9, 10, 8, 7, 11, 9, 10	9·0

Statistical comparison of group means by modified t-test (Bailey, 1959)
Groups 1 and 2 : $d = 3{\cdot}37$ $f = 8$ $P = 0{\cdot}01$
4 and 2 : $d = 3{\cdot}24$ $f = 10$ $P = <0{\cdot}01$

In the second experiment primary transplants to the foot grew more slowly than those to the flank even though the original cell dose was ten times as great (Table II Groups 1, 6), but when the foot bearing a primary transplant was amputated and 10^5 viable cells were re-inoculated to the flank the secondary transplants grew even more rapidly than primary flank transplants (Table II Groups 1, 6; Fig. 2). Injection of *C. parvum* 2 days prior to the re-inoculation significantly inhibited the growth of the secondary transplant (Table II Groups 6, 7; Fig. 2).

In this experiment incubation of tumour cells with ATG before transplantation did not inhibit, indeed if anything it appeared to facilitate, subsequent tumour growth in untreated mice, but it did markedly potentiate the effect of treating the host with *C. parvum*. This is not apparent 40 days after transplantation but after 56 days the synergistic effect of combining the two procedures is strikingly demonstrated (Table II Groups 1, 2, 4, 5; Fig. 3).

In the third experiment pre-incubation of the cells with ATG without C′, though it did not kill the cells, significantly inhibited subsequent tumour growth, and was in effect roughly equivalent to reducing the dose of untreated viable cells by a factor of 10 (Table III Groups 1, 2, 6; Fig. 4, 5). Pre-incubation with ATG plus C′ (Table III Group 7), which resulted in virtually 100 per cent cell death as judged by a dye exclusion test, was followed by growth of a tumour in only one mouse in the group. In the others small nodules appeared but by Day +58 were still only just palpable (about 1–2 mm. diameter).

The inhibitory effect of *C. parvum* alone was confirmed (Table III Groups 4, 5; Fig. 5, 6). The effect of treating the cells with ATG and the host with *C. parvum* (Table III Groups 8, 9; Fig. 5, 6) was even more dramatic than in the previous experiment and was roughly equivalent to reducing the dose of viable cells in control animals Table III Group 3; Fig. 4) by a factor of 100. Once again W2 was the more effective of the two preparations of *C. parvum* which were tested (Table III Groups 4, 5, 8, 9; Fig. 5, 6).

DISCUSSION

The results confirm in one particular model the hypothesis which the experiments were designed to test, namely, that the antitumour effect of heterospecific

TABLE II.—*Second Experiment—see also Fig.* 2 *and* 3

Group	No. of mice	Experimental procedure: Tumour	Experimental procedure: C. parvum	Mean diameter of first tumour on Day +40: Observed values mm.	Group mean	Mean diameter of first tumour on Day +56: Observed values mm.	Group mean	Mean diameter of second tumour 33 days after re-inoculation: Observed values mm.	Group mean
1	6	10^5 untreated viable tumour cells s.c. to L. flank on Day 0	Nil	6, 12, 0, 7, 12, 10	7·8	K†, K, 2, K, K, K	—	—	—
2	6		0·2 ml. i.p. on Day −2	0, 1, 4, 3, 3, 7	3·0	0, 19, 12, 18, 18, 17	14·0	—	—
3	6		0·2 ml. i.p. on Days −2, +3	3, 9, 8, 3, 7, 4	5·5	16, K, K, 14, K, 15	—	—	—
4	6	10^5 pre-incubated* viable tumour cells s.c. to L. flank on Day 0	Nil	12, 12, 10, 11, 2, 9	9·3	K, K, K, K, 2, K	—	—	—
5	6		0·2 ml. i.p. on Day −2	3, 3, 3, 3, 3, 5	3·3	2, 1, 8, 2, 2, 12	4·5	—	—
6	6	10^6 untreated viable tumour cells s.c. to L. foot on Day 0. Foot amputated and 10^5 viable tumour cells reinoculated s.c. to R. flank on Day +40	Nil	6, 8, 4, 3, 3, 5	4·8	—	—	18, 13, 17, 11, 10, 17	12·7
7	12		0·2 ml. i.p. on Day +38	4, 3, 3, 7, 6, 5, 4, 1, 4, 3, 2, 4	3·8	—	—	7, 10, 5, 11, 14, 6, 12, 8, 6, 10, 7, 8	8·7

Statistical comparison of Group Means by t-test.
Groups 1 and 2 on Day +40. Modified t-test (Bailey, 1959): $d = 2 \cdot 65$, $f = 8$, $P < 0 \cdot 05$.
Groups 6 and 7, Second tumour 33 days after re-inoculation. Standard test: $t = 2 \cdot 62$, $f = 16$, $P < 0 \cdot 02$.
* Pre-incubation was performed by incubating $2 \cdot 10^7$ cells in 2·6 ml. Dulbecco's solution containing 6·5 mg. ATG.
† The entry K means the animal had been killed because the tumour had attained a mean diameter of >20 mm.

TABLE III.—*Third Experiment—see also Fig. 4, 5, and 6*

Group	No. of mice	Tumour cells: Untreated or Pre-incubated*	Tumour cells: Dose	*C. parvum* Preparation and dose	Mean tumour diameter on Day 21: Observed values mm.	Mean tumour diameter on Day 21: Group mean mm.	Mean tumour diameter on Day 42: Observed values mm.	Mean tumour diameter on Day 42: Group mean mm.
1	7	Untreated	10^6	Nil	13, 12, 13, 14, 1, 5, 13	10·1	K†, K, K, K, 11, 12, K	—
2	7	Untreated	10^5	Nil	6, 3, 1, 7, 6, 4, 6	4·7	20, 18, 17, K, K, 21, 21	—
3	6	Untreated	10^4	Nil	1, 0, 0, 1, 0, 0	0·3	8, 14, 0, 0, 10, 10	7·0
4	7	Untreated	10^6	W2. 0·2 ml. s.c. on Day −2	2, 2, 5, 2, 0, 9, 2	3·1	13, 19, 22, 16, 13, 22, 18	18·1
5	6	Untreated	10^6	P2. 0·2 ml. s.c. on Day −2	8, 5, 2, 6, 5, 5	5·2	21, 20, 17, 17, 22, 18	19·2
6	7	Pre-incubated ATG without C′	10^6	Nil	1, 3, 0, 2, 0, 7, 4	2·4	22, 19, 20, 18, K, K, 18	—
7	7	Pre-incubated ATG with C′	10^6	Nil	0, 0, 0, 0, 0, 0, 0	0·0	2, 2, 1, 0, 1, 0, 8	2·0
8	7	Pre-incubated ATG without C′	10^6	W2 0·2 ml. s.c. on Day −2	0, 0, 0, 1, 0, 0, 0	0·1	0, 9, 1, 3, 16, 6, 5	5·7
9	7	Pre-incubated ATG without C′	10^6	P2. 0·2 ml. s.c. on Day −2	0, 1, 0, 4, 0, 1, 0	0·9	5, 20, 7, 18, 0, 12, 7	9·9

* Pre-incubation was performed by incubating 2×10^7 cells in 2·6 ml. Dulbecco's solution containing 6·6 mg. ATG. In group 7 only, 0·65 ml. undiluted serum was added as a source of C′.

† The entry K means the animal had been killed because the tumour had attained a mean diameter of >20 mm.

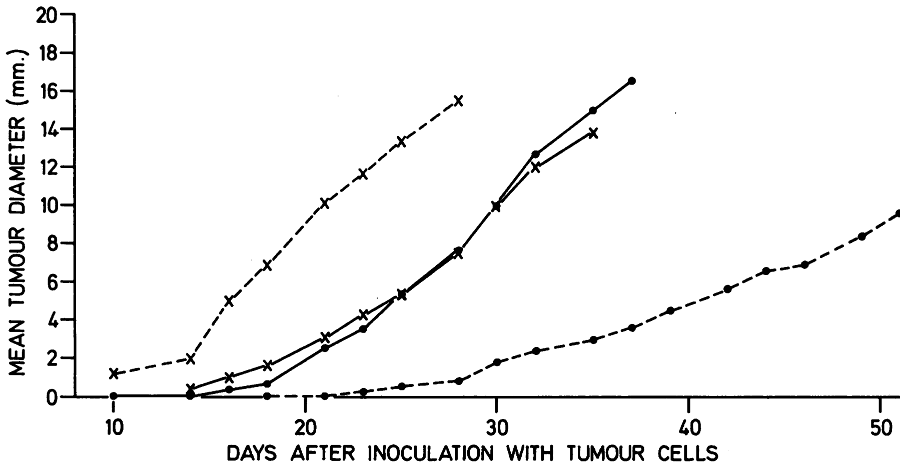

FIG. 5.—Third experiment. Separate and combined effects on tumour growth of (1) i.p. injection of the host with *C. parvum* (W2) and (2) pre-incubation of tumour cells with ATG (Batch E5Y1).

×············× No treatment.
×————× *C. parvum.*
●————● Pre-incubation with ATG.
●············● *C. parvum* and pre-incubation.

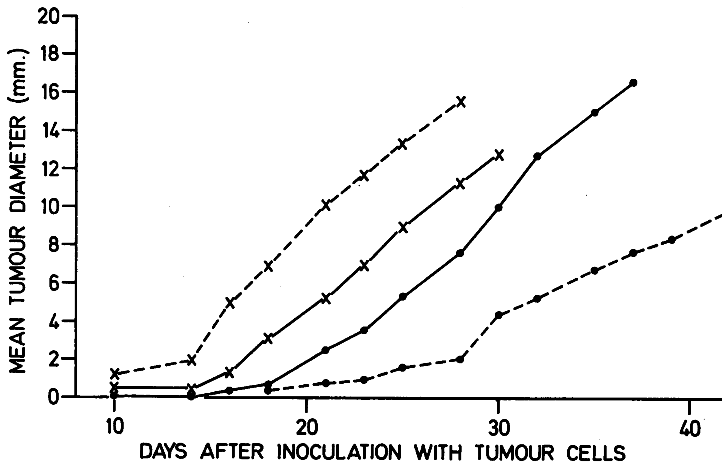

FIG. 6.—Third experiment. Separate and combined effects on tumour growth of (1) i.p. injection of the host with *C. parvum* (P2) and (2) pre-incubation of tumour cells with ATG (Batch E5Y1).

×············× No treatment.
×————× *C. parvum.*
●————● Pre-incubation with ATG.
●············● *C. parvum* and pre-incubation.

antibody can be markedly potentiated by a procedure which stimulates phagocytic activity.

It remains to be seen whether this conclusion is of general validity or whether the findings were determined by special features of the particular model chosen, including the type of tumour and the strain of mouse. For example, A/HeJ mice, being deficient in C′5, are presumably more dependent upon phagocytosis as a means of immunological defence than mice which are not so deficient, and it is therefore conceivable that they would show a relatively greater response to stimulation by *C. parvum.*

To test this we have set up experiments similar to those described with transplants of chemically induced sarcomas in A/HeJ, CBA and C57BL mice. We are also studying the effect of treating the tumour bearing animals with ATG and *C. parvum* instead of treating the tumour before inoculation with ATG and the animals with *C. parvum* as in the present investigation. It would seem premature to consider possible clinical applications until the results of these additional experiments are available.

We gratefully acknowledge generous gifts of *C. parvum* preparations from the Wellcome Foundation (Dr. J. Cameron) and from Professor Marcel Raynaud of the Pasteur Institute, Paris. We are indebted also to Mrs. Evelyn Pawley for skilled technical assistance.

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

REFERENCES

ANDERSON, N. F., JAMES, K. AND WOODRUFF, M. F. A.—(1967) *Lancet*, i, 1126.

BAILEY, N. T. J.—(1959) ‘ Statistical Methods in Biology ’. London (English Universities Press).

BORSOS, T. AND COOPER, M.—(1961) *Proc. Soc. exp. Biol. Med.*, **107**, 227.

CINADER, B., DUBISKI, S. AND WARDLAW, A. C.—(1964) *J. exp. Med.*, **120**, 897.

HALPERN, B. N., PREVOT, A.R., BIOZZI, G., STIFFEL, C., MOUTON, D., MORARD, J. C., BOUTHILLIER, Y. AND DECREUSEFOND, C.—(1963) *J. Reticulo-endothelial Soc.*, **1**, 77.

NILSSON, U. R. AND MÜLLER-EBERHARD, H. J.—(1967) *J. exp. Med.*, **125**, 1.

SMITH, LINDSAY H. AND WOODRUFF, M. F. A.—(1968) *Nature, Lond.*, **219**, 197.

WOODRUFF, M. F. A. AND BOAK, J. L.—(1966) *Br. J. Cancer*, **20**, 345.

WOODRUFF, M. F. A. AND SMITH, LINDSAY H.—(1970) *Nature, Lond.*, **225**, 377.