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TUMOUR GROWTH AND NON-SPECIFIC IMMUNITY IN RATS: THE MECHANISMS INVOLVED IN INHIBITION OF TUMOUR GROWTH

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Summary.—Various non-specific *in vivo* stimulants of phagocytosis, such as peptone, starch, glycogen, BCG, inhibit the growth of Walker carcinosarcoma in rats. This was confirmed by comparison of the histological appearance of tumour beds and tumours of peptone-treated and control rats. In rats given peptone or BCG, tumour inhibition was detectable only during a limited period of time. Experiments on the effect of pretreatment with peptone on the growth of Walker ascites tumour cells revealed a clear-cut inhibition, and suggest that tumour cells may be successfully eliminated during the lag phase. Data showing that activated macrophages labelled with ^{51}Cr significantly accumulate around the tumour implant support the view that macrophages are of prime importance in the elimination of tumour cells in this model system.

It is now generally accepted that many neoplasms are capable of eliciting an immune response in the host and that the animal's immune defence system crucially affects its response to carcinogens and transplanted cancer cells. According to this view, the development of a cancer cell clone into a tumour is associated with some failure of the immune system. However, the observations that neoplasms with high antigenicity can grow unchecked (Humphreys *et al.*, 1962; Old *et al.*, 1962; Potter, Hoskins and Oxford, 1969) suggest that even these tumours might have insufficient immunogenic potential to induce their own destruction.

Other observations have shown that non-specific stimuli may also inhibit tumour growth (Yashphe, 1971). In *Nippostrongylus brasiliensis*-infected rats, the growth of Walker carcinosarcoma is either markedly suppressed or greatly enhanced, depending on the timing of the inoculation of the tumour cells in relation to the parasite infection (Keller, Ogilvie and Simpson, 1971). Thus, in this model system both inhibition and enhancement can be studied under controlled conditions. Further work has indicated that in the same tumour system peptone also suppresses tumour growth and that activated macrophages are instrumental in the elimination of tumour cells (Keller and Jones, 1971; Keller, 1972). The present work confirms and extends these observations by showing that many non-specific stimulants known to activate macrophages may inhibit tumour growth when given in an appropriate time interval in relation to the inoculation of the tumour cells. This effect was also confirmed by comparing the histology of tumours and tumour beds in peptone-treated rats and controls. The important role of macrophages in tumour cell elimination was further substantiated by the demonstration of a selective accumulation of labelled macro-

phages in the vicinity of tumours and of the inability of polymorphonuclear leucocytes to eliminate tumour cells *in vitro*.

MATERIALS AND METHODS

Animals.—Colony bred Osborne–Mendel or CNRS rats (weight 180–220 g) were used for most *in vivo* experiments. To quantitate the macrophage traffic in the vicinity of tumours, inbred rats of the DA strain were used. For *in vitro* experiments, glycogen-induced peritoneal cells were obtained from inbred Lewis donor rats.

Tumour.—Walker 256 carcinosarcoma ascites cells were maintained by serial passage in the homologous rat strain. The cells were washed 3 times and their viability judged by the trypan blue exclusion test. For most *in vivo* experiments, rats were given subcutaneously 10^7 tumour cells, and tumour weights were recorded 10 days later. For *in vitro* experiments, 5×10^4 tumour cells were added to each Falcon dish.

To obtain an insight into the sequence of tumour cell elimination, 40 Osborne–Mendel rats were given 15 ml of 10% peptone i.p. (Fluka AG, Buchs SG) on Day −3. On Day 0, these rats and 40 untreated controls were given 10^6 Walker tumour cells i.p., and the number of rats dying until Day 50 was registered.

In another experiment, 36 Osborne–Mendel rats were given 15 ml of 10% peptone i.p. on Day −3. On Day 0, these rats and 36 untreated rats were given 10^6 Walker carcinosarcoma cells i.p. On Days 1, 2, 3, 4, 5 and 6, 6 rats of either group were sacrificed, the peritoneal cells harvested and the number of Walker carcinosarcoma cells counted.

Treatment of rats in vivo.—Peptone (10%; 15 ml; Fluka AG, Buchs SG), glycogen (0·1%; 20 ml; Fluka AG, Buchs SG), starch (3%; 20 ml; Fluka AG, Buchs SG), casein (7%; 15 ml; Fluka AG, Buchs SG), bacillus Calmette–Guérin (BCG; 40×10^6 organisms per animal; Swiss Serum Institute), Freund's complete adjuvant (FCA; 0·5 ml; Difco) or endotoxin (60 μg; lipopolysaccharide B *Esch. coli* 026 : B6; Difco) were injected once i.p. In most of the experiments, these agents were injected 5 days before inoculation of tumour cells. For some of the agents, namely peptone and BCG, other time intervals between injection of the irritant and inoculation of tumour cells were additionally examined. Poly I/Poly C (500 μg/animal on Days −3, −1 and +2; Calbiochem) and Poly A/Poly U (25 μg/animal on Days −3 and +2; Sigma) were injected i.p. several times.

Histology.—Ten rats were given 15 ml of 10% peptone i.p. 5 days before the inoculation of 10^7 Walker carcinosarcoma cells by subcutaneous route. A control group of 10 rats received the same number of tumour cells without pretreatment with peptone. Biopsies of solid tumour and/or subcutaneous tissue were taken from the site of tumour cell inoculation after an interval of 10 days. The tissue samples were fixed in Carnoy's fluid, taken through alcohol into methylbenzoate and embedded in paraffin. Sections of 4 μm were stained with haemalum–eosin.

Labelling of macrophages with ^{51}Cr.—To quantitate the macrophage traffic in the vicinity of tumours, peritoneal cells taken 3–4 days after an i.p. injection of peptone into inbred DA rats were washed 3 times with Hank's balanced salt solution (HBSS). The cells were labelled by incubation with sodium chromate (^{51}Cr; specific activity 112–122 mCi/mg Cr; Eidgenössisches Institut für Reaktorforschung, Würenlingen) at 200 μCi per 30–36 $\times 10^8$ cells for 60 minutes at 37°. After thorough washing, 2×10^7 labelled donor macrophages were injected i.p. into recipient DA rats. Recipients were either untreated controls or had been inoculated s.c. with 10^7 Walker carcinosarcoma cells 48 hours before. One, 2, 3, 4, 5 and 7 days after the injection of labelled macrophages, 10 rats of each group were killed. The tumours and tumour beds as well as normal tissue samples of equal size and weight were excised. Peritoneal cells, mesenteric and inguinal lymph nodes, small intestine, thymus, spleen and liver were also removed. Assay of radioactivity was performed in a well-type scintillation counter and the distribution of radioactivity of different sites, and in normal and tumour-bearing rats, was compared.

Polymorphonuclear leucocytes (PMNs) were obtained 4–5 hours after i.p. injection of 15 ml of 0·1% glycogen (Keller *et al.*, 1968). Cultures were prepared by seeding 1–5 $\times 10^6$ polymorphonuclear leucocytes into Falcon petri dishes. After 1 hour of incubation at 37°, the medium was decanted and 2 ml of medium 199 (Serva GmbH, Heidelberg) supplemented with penicillin (50 u/ml), streptomycin 50 μg/ml) and 10% heat-inactivated (120 minutes at 56°) rat serum were added. Immediately afterwards, 5×10^4 viable Walker tumour cells

were added. After 2, 4 and 5 hours of incubation, the number and viability of tumour cell samples removed from the dishes were assessed and the cells remaining in the dishes were microscopically examined, as described for macrophages (Keller and Jones, 1971; Keller, unpublished).

RESULTS

Effect of various agents on tumour growth in vivo

Results of experiments on the effect of various agents on tumour growth *in vivo* are summarized in Table I. Since tumour weights in untreated controls

TABLE I.—*Effect of Pretreatment with Various Agents on Tumour Growth (10^7 Tumour Cells were Inoculated s.c. on Day 0)*

Treatment of rats	Day of treatment	Relative tumour weight (non-treated controls =100%; 15 rats per group)
Peptone	−5	30/21/24
Starch	−5	36/40
Casein	−5	53/60
Glycogen	−5	45/55/24
FCA	−5	66/56
BCG	−5	13/27/63
Endotoxin	−5	225/70/47/48
Poly I/Poly C	−3, +1, +2	35
Poly A/Poly U	−3, +2	53

varied to some degree from one experiment to the other, the percentage of the mean weights of the tumours is presented. In these experiments, most agents were given only once, *i.e.* 5 days before the inoculation of tumour cells, using 15 rats per group. The data show that control rats always bore easily detectable tumours and that the individual tumour weights showed little scattering. In contrast, in many of the rats pretreated with peptone, starch, casein, glycogen, BCG or FCA no tumour could be detected on Day 10, and in rats pretreated with these agents and bearing tumours, the scattering of the tumour weights was generally much more pronounced than in controls. Under the various agents tested, peptone suppressed tumour growth most effectively and regularly. The greatest scatter followed the administration of endotoxin; this was observed in 4 different experiments. Poly I/Poly C produced a marked suppression in tumour weight whereas Poly A/Poly U in the low dose range used was less effective.

Histology of tumours and tumour beds in controls and in rats pretreated with peptone

In peptone-treated rats given 10^7 Walker carcinosarcoma cells by the s.c. route, 6 out of 10 developed no macroscopically detectable tumour within 10 days

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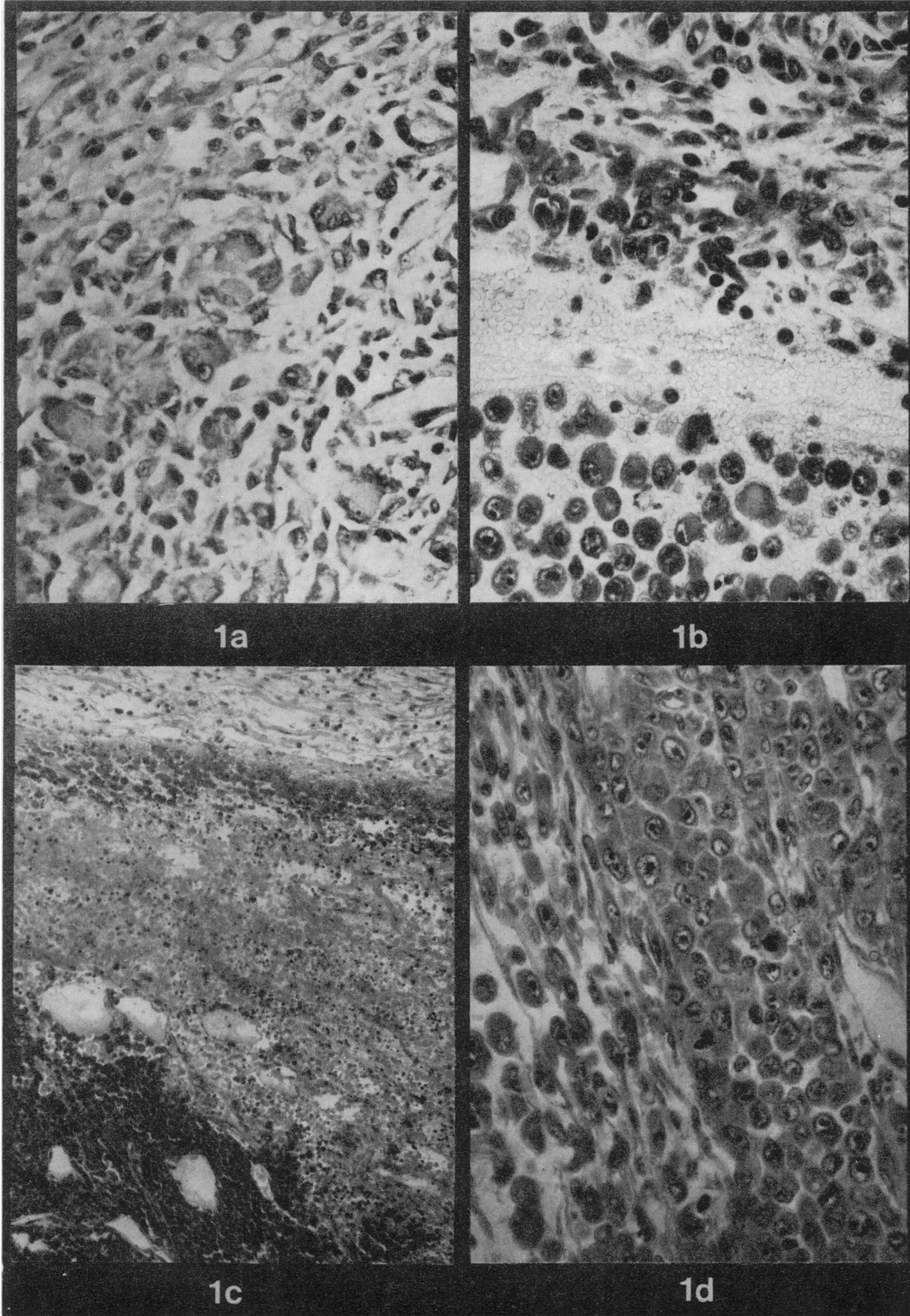
EXPLANATION OF PLATE

FIG. 1.—Subcutaneous tissue taken from the site of the implant 10 days after the inoculation of 10^7 Walker carcinosarcoma cells. Rats were either pretreated with peptone (A–C) or untreated controls (D). Paraffin sections, 4 μm, haemalum–eosin. (A) no tumour growth in peptone-treated rats. Abundant formation of granulation tissue at inoculation site ($\times$ 375). (B, C) tumour growth in peptone-treated animals. Peripheral demarcation of tumour mass by granulation tissue (B $\times$ 375) or necrosis (C $\times$ 95). (D) tumour growth in controls. No apparent host reaction at periphery of tumour ($\times$ 375).


1a
1b
1c
1d

after inoculation. After this time interval, the remaining 4 animals of the experimental group and all 10 non-treated controls were bearing tumours of 20 g or more (average in controls 25 g). Histologically, the differences between untreated controls and the experimental group were striking. The subcutaneous tissue of some of the peptone-treated rats with no detectable tumour was granulomatous and no tumour cells could be found (Fig. 1A), while in others remnants of small tumour masses were surrounded by mononuclear, in part phagocytic, cells (Fig. 1B). In peptone-treated rats with growing tumours, peripheral and central necrosis of the tumour was evident; peripheral infiltration with mononuclear elements was less pronounced than in the group with no tumour growth (Fig. 1C). In marked contrast, signs of active tumour growth with no or minimal reaction of the host were observed in non-treated controls; here, only central tumour necrosis was noted (Fig. 1D).

Time course of the tumour inhibitory effect induced by peptone and BCG

Since earlier (Yashphe, 1971; Keller and Jones, 1971) and present data have shown that host resistance to tumours may be increased by non-specific stimula-

TABLE II.—*The Percentage of Tumour Growth* in vivo *Following Treatment with Peptone or BCG* (*Controls* 100%)

Treatment of rats	Days of treatment (inoculation of tumour cells on Day 0)					
	−30	−10	−5	0	5	10
Peptone	95	85/84	30*/21*/24*	54*/25*	12*/48*/55*	100
BCG	106/100	110	13*/27*/63*	—	105/66*	—

Each value represents the mean of a group of 15 rats.
* Significantly lower than in controls.

TABLE III.—*Death Rate of Osborne–Mendel Rats Inoculated i.p. with* 10^6 *Walker Carcinosarcoma Cells on Day* 0

Day after tumour cell inoculation	Treatment: None	Treatment: 15 ml 10% peptone i.p. on Day −3
7	4	—
8	2	—
9	4	—
10	2	—
11	3	—
12	3	—
13	5	1
14	4	1
15	3	2
16	1	—
17	1	—
18	1	2
19	—	2
20	2	2
21	—	—
22	—	—
23	—	1
24	—	2
25	—	1

Each experimental group consisted of 40 rats. On Day 50, 5 controls (12%) and 26 rats pretreated with peptone (65%) had survived.

tion, it seemed of interest to characterize the time course of this effect. So far, only peptone and BCG have been studied in detail (Table II). The results show that to be effective, peptone and BCG must be given a few days before or after tumour-cell inoculation, and that beyond these narrow limits no inhibition could be elicited.

Time course of tumour cell elimination in vivo

First, the effect of peptone on Walker carcinosarcoma ascites cell growth was examined. Table III shows that treatment with peptone greatly reduced the mortality (65% survivals) in comparison to untreated controls (12% survivals). Moreover, in animals showing tumour growth despite peptone treatment, the survival time was significantly prolonged.

When the number of Walker cells which were present in peritoneal cells harvested 1–6 days after the inoculation of 10^6 tumour cells i.p. was followed, a similar clear-cut difference was seen (Table IV). In controls, Walker cells were always

TABLE IV.—*The Number of Walker Ascites Tumour Cells* ($\times 10^6$) *Recovered from the Peritoneal Cavity after Inoculation of* 10^6 *Tumour Cells on Day* 0

	Untreated controls	Pretreated with peptone on Day −3
Day 1	0·005 (±0·008)*	0
Day 2	0·038 (±0·080)	0·017 (±0·041)
Day 3	5·147 (±2·001)	0·183 (±0·259)
Day 4	29·157 (±8·99)	0·022 (±0·024)
Day 5	44·70 (±18·26)	0·188 (±0·308)
Day 6	64·67 (±21·55)	0·85 (±1·65)

* Each value represents the mean of 6 rats.

present on Day 2 following inoculation whereas in some of the rats pretreated with peptone no tumour cells could be found until Day 6. Whereas the mean number of tumour cells in controls showed a constant increase, the mean number of tumour cells remained low in rats pretreated with peptone (Table IV).

Accumulation of macrophages in the vicinity of tumours

On Days 1–7 after the injection of labelled macrophages (*i.e.* Days 3–9 after tumour cell inoculation), the levels of radioactivity in the tumours and tumour beds were always significantly ($P < 0{\cdot}001$) higher (Fig. 2). The data thus show that ^{51}Cr-labelled macrophages congregate at the tumour site.

When the migration of macrophages to other areas such as inguinal and mesenteric lymph nodes, small intestine, spleen, liver and thymus was compared in controls and in tumour-bearing rats, no clear-cut differences in homing were observed. However, in some but not all of the experiments, the number of labelled macrophages in peritoneal cell samples of tumour-bearing rats were markedly depressed by Day 3; this may indicate a more rapid withdrawal of cells from the peritoneal cell pool.

In vitro *experiments with polymorphonuclear leucocytes*

When Walker ascites tumour cells were added to cultures consisting of more than 90% of polymorphonuclear leucocytes, their number remained constant

and their viability (assessed by the trypan blue exlusion test) was not affected during the first 5 hours of incubation. Examination of the cells remaining in the dishes gave no indication of phagocytosis of tumour cells by PMNs.

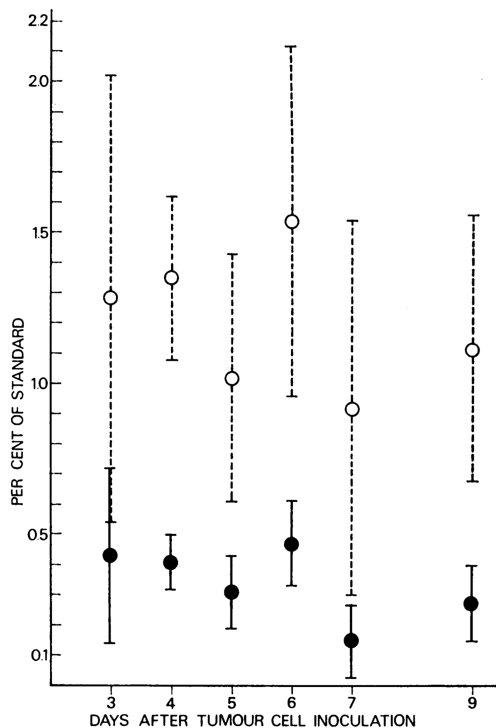


FIG. 2.—Accumulation of radioactivity at the site of tumours. ● Range of radioactivity in the skin of controls and from the opposite side of tumour-bearing rats. ○ Range of radioactivity at the tumour site. Each value represents the mean of 10 rats.

DISCUSSION

There is growing evidence that various agents with a wide range of properties stimulate immunological reactivity non-specifically and also inhibit tumour growth (Yashphe, 1971). The observations that reagin formation induced by helminths in rats and inhibition of tumour growth are both thymus-dependent (Wilson *et al.*, 1967; Ogilvie and Jones, 1967; Metcalf, 1966) led to investigations into the effect of nematode infection on tumour growth in mice and rats (Keller *et al.*, 1971). These experiments have shown that not only is the growth of Walker carcinosarcoma either greatly inhibited or stimulated in rats infected with the nematode, *Nippostrongylus brasiliensis*, depending on the timing of the inoculation of the tumour cells in relation to the parasite infection, but also that tumour inhibition is a cellular phenomenon whereas tumour enhancement is due to a factor appearing in the serum (Keller and Jones, 1971).

Present work has shown that, among other agents tested for inhibition of tumour growth, peptone was found to be most effective (Table I). Both the

histological findings and the demonstration of a selective accumulation of activated macrophages in the vicinity of tumours suggest that the host's capacity to overcome tumour growth, as reflected by the infiltration of mononuclear cells, is greatly stimulated by peptone pretreatment. Some of the other agents found to be capable of suppressing tumour growth in the present system have been shown to inhibit tumour growth in other experimental systems (Yashphe, 1971), and to stimulate phagocytosis (Roos, 1970).

Thus, the recent observations that inhibition of tumour growth was due to radioresistant cells (Keller and Jones, 1971; Keller, 1972), that addition of tumour cells to a monolayer of activated macrophages resulted in a rapid and marked decrease in the percentage of tumour cells remaining in culture (Keller and Jones, 1971), that microscopic examination of the cells remaining in the dishes showed a large number of tumour cells partly or entirely contained within engulfing macrophages (Keller and Jones, 1971; Keller, 1972) and that the phagocytic capacity of the cells was greatly reduced by pretreatment with anti-macrophage serum but not by anti-thymocyte serum (Keller, 1972), are all in keeping with the present findings and suggest that, in the model system under discussion, the mononuclear phagocytes are of prime importance for inhibition of tumour growth.

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