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EVIDENCE FOR COMPROMISE OF TUMOUR IMMUNITY IN RATS BY A NON-SPECIFIC BLOCKING SERUM FACTOR THAT INACTIVATES MACROPHAGES

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Summary.—A factor in the serum of rats infected with the nematode parasite, *Nippostrongylus brasiliensis*, enhances the growth of Walker carcinosarcoma cells in rats *in vivo* and also fully reverses inhibition of tumour growth achieved by cellular mechanisms augmented by nematode infection or by RES stimulants. Under specified *in vitro* conditions, the blocking rat serum factor largely counteracts the elimination of tumour cells in the rat system and also in a mouse effector/target cell system. Moreover, activated peritoneal macrophages cultured *in vitro* manifest various signs of inactivation upon addition of the serum from rats infested with the parasite.

EVIDENCE for the important role of specific cellular immune processes in the elimination of tumour cells has increased impressively (Hellström and Hellström, 1969; Klein, 1969; Good and Finstad, 1969; Burnet, 1970*a*,*b*), but there is little to account for the often observed discrepancy that the host can successfully reject homografts and yet fails to overcome tumours. Certain individuals may carry progressively growing neoplasms even though their lymphoid cells are able to exert a specific cytotoxic effect on their own tumour cells *in vitro* (Hellström *et al.*, 1971*a*); this process is frequently blocked by the sera of tumour-bearing individuals (Hellström *et al.*, 1971*b*). Thus, in these tumour systems the development of specific serum blocking activity directed against cytotoxic lymphocytes may be involved in the failure to control tumour growth. We have evidence that in the *Nippostrongylus brasiliensis*-infected rat, the growth of Walker carcinosarcoma can be either suppressed or enhanced, depending on the timing of the inoculation of tumour cells in relation to the parasite infection (Keller, Ogilvie and Simpson, 1971). Evidence to date indicates that in this (Keller and Jones, 1971; Keller, 1972; Keller and Hess, 1972) and some other model systems (Hibbs, Lambert and Remington, 1972), tumour cell elimination is attributable to activated macrophages not specifically immune against the tumour, whereas enhancement of tumour growth *in vivo* can be transferred with serum from tumour-free rats in which immunity has developed after a nematode infection (Keller, Ogilvie and Simpson, 1971). This serum and its IgG_2 component were found to prevent the engulfment of tumour cells spread on a macrophage monolayer *in vitro* (Keller and Jones 1971). The present work provides further information regarding the mechanisms involved in the counteraction of tumour immunity by the blocking serum factor.

MATERIALS AND METHODS

Animals.—Colony-bred Osborne–Mendel or CNRS rats (weight 180–220 g) were used for *in vivo* experiments. For *in vitro* experiments, rat peritoneal cells were taken from inbred

Lewis or DA rats which had been injected with 10 ml of 10% proteose peptone (Fluka AG, Buchs SG) 3 days earlier. Mouse peritoneal cells were obtained from C3H mice inoculated i.p. with 0·5 ml of complete Freund's adjuvant (FCA; Difco) 7 days previously.

Tumours.—Walker 256 carcinosarcoma ascites tumour cells were maintained and processed as described (Keller and Hess, 1972). For some of the experiments, ascites tumour cells had been incubated repeatedly with normal rat serum or with serum from rats infested with the parasite, then washed 3 times in Hank's balanced salt solution (HBSS), and 10^7 cells injected s.c. Ehrlich ascites tumour cells (EATC) were maintained by serial passage in C3H mice.

Parasite.—The parasite *Nippostrongylus brasiliensis* was maintained by methods previously described (Ogilvie, 1967; Keller, 1970). Colony-bred Osborne–Mendel rats or inbred rats of the Lewis strain with an initial weight of between 180 and 220 g were infected once or several times with larvae according to the immunization schedule described earlier (Keller, 1970). Sera with blocking activity were obtained on Day 30 after a primary infection or 5–10 days after a fourth infection with *N. brasiliensis* (Keller *et al.*, 1971).

Elimination of tumour cells in vitro.—Rat peritoneal fluid containing peptone-induced activated macrophages was harvested after instillation of 10 ml of HBSS. The cells were first washed 3 times with HBSS and then suspended in medium 199 (Serva GmbH, Heidelberg) supplemented with penicillin (50 u/ml), streptomycin (50 μg/ml), and 10% heat-inactivated (120 min 56°) normal rat serum. The peritoneal exudate was generally 70–80% macrophages as judged by morphological criteria and by the uptake of the dye, neutral red; viability was determined by the trypan blue exclusion test. Cultures were prepared by seeding approximately 2×10^6 macrophages into 3·5 cm Falcon plastic petri dishes (Evans and Alexander, 1970). After 15 min incubation, the medium was decanted and 2 ml of supplemented medium 199 was added. After incubation for 120 min at 37°, the dishes were washed intensively with culture medium to remove non-adherent cells. The resulting monolayer consisted predominantly of macrophages and invariably contained less than 5% lymphocytes, granulocytes and mast cells. At this stage, 5×10^4 viable Walker carcinosarcoma cells were added to each dish. The cultures were supplemented either with 10% heat-inactivated normal rat serum or with 10% heat-activated serum from rats infested with *N. brasiliensis* and then incubated at 37° for 2, 4 or 5 hours. The non-adhering cells were then removed by intensive washing of the monolayer with jets of medium 199. At each time interval, the number and viability of tumour and other cells removed from 3 dishes were assessed. The cells remaining in the dishes were fixed with methanol and stained with Giemsa. Mouse peritoneal exudate cells induced by FCA were obtained, washed and cultured as described for rat peritoneal cells. 5×10^4 viable EATC were added to each of the dishes to which the macrophages adhered, and the cultures were incubated for 5 hours at 37°. When the effects of normal rat serum or of serum from *N. brasiliensis*-infested rats were tested in this system, mouse serum was replaced by the same concentration (10%) of one of these sera.

Study of macrophage movement.—The photomicrographic trace technique (Harris, 1953) was used. Peptone-induced rat peritoneal cells were allowed to adhere to slides as described before. After 2 hours, the medium containing normal rat serum was replaced by medium supplemented with 10% serum from *N. brasiliensis*-infested rats, the coverslips sealed with paraffin and then incubated at 37° for a further 2–4 hours. The locomotion of the macrophages was recorded photographically.

To assess whether blocking serum factor activity could be absorbed out by mixing with various tissues, 10 ml of serum from *N. brasiliensis*-infested rats were incubated for 30 min at 37° with either 10^8 Walker carcinosarcoma cells or with freshly harvested and washed organisms of *Nippostrongylus brasiliensis*, *Fasciola hepatica* or *Dicrocoelium dendriticum*. This procedure was repeated 6 times, and the supernatant cell-free serum was then tested in the *in vitro* system.

RESULTS

Reversal of cell-mediated tumour immunity in vivo *by blocking serum factor*

Results of an initial series of *in vivo* experiments, summarized in Table I, show that serum taken from rats 30 days after a primary infection with *N. brasiliensis* fully reverses tumour immunity induced by the parasitic infection 5 days before the tumour cells were inoculated. Similar effects were obtained with serum taken

TABLE I.—*Enhancement by Serum of* N. brasiliensis*-infested Rats of Tumour Growth in CNRS Outbred Rats*

Group no. (15 rats/ group)	Treatment of recipients	Mean weight of tumour ± S.D. (g)
1	Tumour only	6·18 (±1·6)
2	Infected with *N. brasiliensis* 5 days before tumour	0·68 (±0·15)*
3	Infected with *N. brasiliensis* 5 days before tumour + normal rat serum	2·19 (±0·45)*
4	Infected with *N. brasiliensis* 5 days before tumour + normal rabbit serum	1·64 (±0·42)*
5	Infected with *N. brasiliensis* 5 days before tumour + Day 30 *N. brasiliensis* serum	8·93 (±1·85)
6	Day 30 *N. brasiliensis* serum + tumour	10·4 (±2·1)†
7	Tumour cells adsorbed with Day 30 *N. brasiliensis* serum before inoculation	9·6 (±1·95)†
8	Peptone i.p. 3 days before tumour	1·98 (±0·65)*
9	Peptone i.p. 3 days before tumour + Day 30 *N. brasiliensis* serum	7·24 (±0·98)

2 ml of each of the various sera were injected i.p. on Days −5, −2, 0, +2 and +5. Recipients of each group were inoculated with 10^7 Walker carcinosarcoma cells on Day 0 and tumours were weighed 10 days later.

* Significantly lower than for Group 1 (Student's *t*-test; $P < 0·001$).
† Significantly higher than for Group 1 (Student's *t*-test; $P < 0·001$).

8 days after a fourth infection with *N. brasiliensis*. Normal serum from rats or rabbits gave no such effects although normal rat serum produced a slight reversal of tumour inhibition. Serum from parasitized rats also fully reversed peptone-induced inhibition (Table I).

When Walker ascites tumour cells which had been mixed with serum from rats infested with the parasite were then injected subcutaneously into CNRS rats, tumour growth was significantly enhanced (Table I) in comparison with controls. These data thus show that enhancing activity is present in the serum of rats immune to *N. brasiliensis* and that the factor responsible for such activity may be adsorbed on to the tumour cells.

In vitro *inactivation of macrophages by blocking serum factor*

Recent work (Keller and Jones, 1971) has shown that the engulfment of tumour cells by a monolayer of activated macrophages taken from nematode-infected or peptone-treated rats is prevented in the presence of serum from parasite-infested rats. To determine whether this serum effect was exerted on macrophages or on tumour cells, or whether both were affected, the morphological

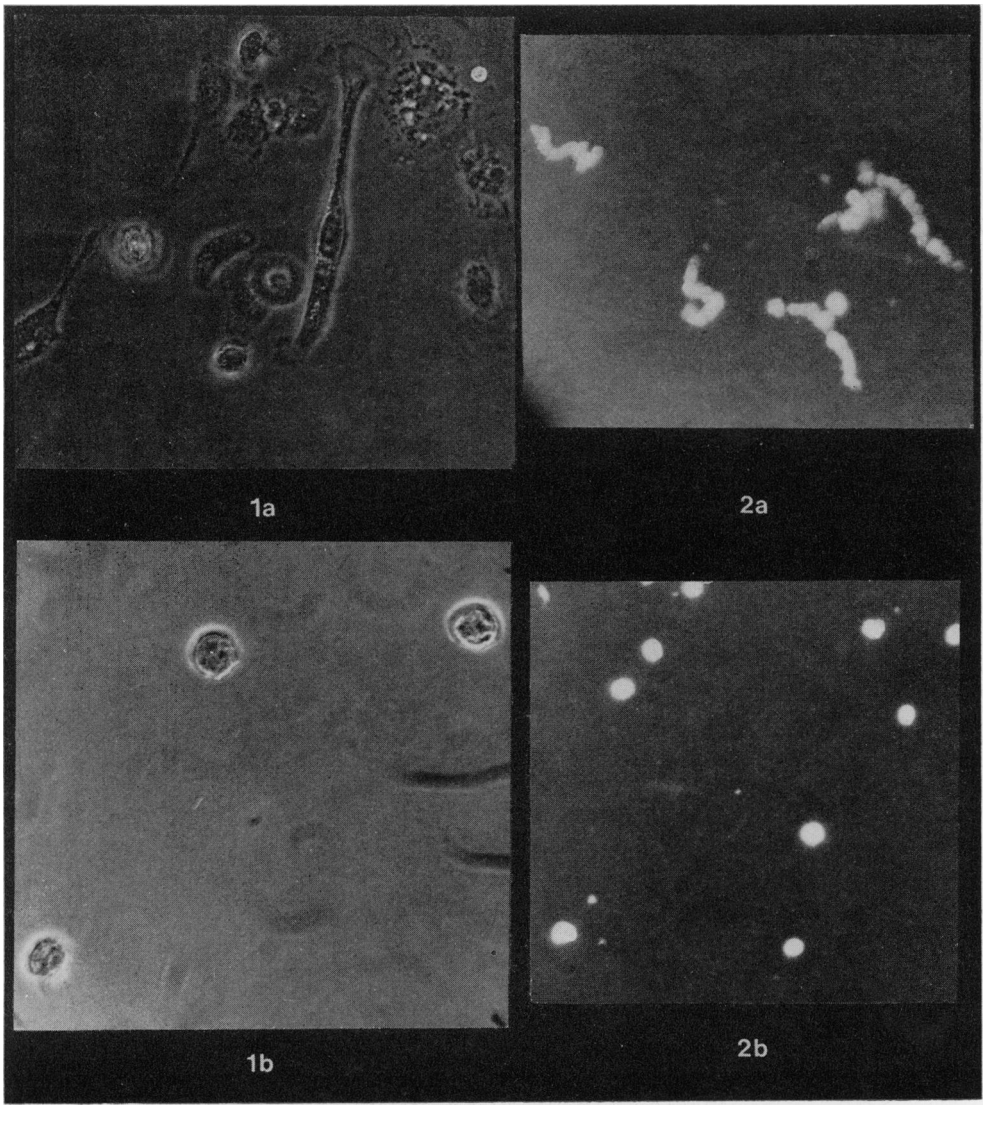
EXPLANATION OF PLATES

FIG. 1a.—Phase contrast photomicrograph (×625) of peptone-induced rat macrophages mixed with normal rat serum; note particularly the cytoplasmic extensions and ruffled membranes.

FIG. 1b.—Phase contrast photomicrograph (×625) of peptone-induced rat macrophages mixed with serum from *N. brasiliensis*-infested rats; the cells are rounded up and are immobile.

FIG. 2a.—Locomotion of peptone-induced rat macrophages which had been mixed with normal rat serum (×250; exposure 20 min).

FIG. 2b.—Immobility of peptone-induced rat macrophages which had been mixed with serum from *N. brasiliensis*-infested rats (×250; exposure 50 min).


1a
2a
1b
2b

characteristics of these cells were followed for 4 hours after incubation with 10% serum from *N. brasiliensis*-infested rats *in vitro*. Neither the morphology nor the growth of the tumour cells was affected in the presence of the worm serum. However, activated macrophages were markedly altered by the serum of worm-infested rats: the cytoplasmic extensions disappeared, the ruffled membrane movements ceased, the cells became rounded up and their ability to adhere to the dishes was diminished without showing signs of cellular damage (Fig. 1a, 1b). These changes were already detectable after 2 hours and were fully developed by 4 hours of incubation.

Consequences of macrophage inactivation by serum blocking factor

To learn more about which macrophage functions might be primarily affected by blocking serum factor, the peptone-induced migration of macrophages into the peritoneal cavity *in vivo* was followed (a) in untreated controls, (b) in rats given 2 ml of worm serum i.p. on Days 0, +1 and +3 and (c) in rats which had been infested with 4000 *N. brasiliensis* larvae 30 days earlier. On Days 3, 4 and 6 after the peptone injection, 15 rats of each group were sacrificed, the peritoneal cells harvested, the total number of macrophages was assessed and the percentage of macrophages which could be washed off after 2 hours culture at 37° was determined. The results obtained on Day 3 after i.p. injection of peptone are given in Table II. The data show that the number of macrophages migrating into the peritoneal cavity was similar in the 3 experimental groups. Thus, the irritant-induced migration of macrophages is affected neither by the natural presence nor the passive administration of serum in response to the parasite. Similar results were obtained with cells harvested on Days 4 and 6 after the injection of peptone. However, evaluation of the capacity of these macrophages to adhere to the surface of the culture dishes, as determined by the number of cells which could be washed off after 2 hours, consistently revealed clear differences. The data in Table II show that in controls only about 15% of the adhering cells were washed

TABLE II.—*Contrast between the Effect of Blocking Serum on the Adherence of Macrophages* in vitro *and on Migration* in vivo (15 *Rats per Group*)

	Treatment of rats		
	Peptone only (controls)	Peptone and blocking serum i.p.	Peptone given to worm-infested rats
Number of peritoneal macrophages ($\times 10^6$) on Day 3 after peptone	12·62 (±1·97)	11·47 (±2·04)	15·24 (±1·89)
	(1)*	(2)*	(3)*
Number of macrophages ($\times 10^6$) washed off after 2 hours culture at 37° *in vitro*†	0·236 (±0·053)	1·274 (±0·315)	0·508 (±0·139)

* Student's *t*-test (1) *vs* (2) $P < 0{\cdot}001$.
(1) *vs* (3) $P < 0{\cdot}001$.

† 2×10^6 macrophages had been added to each dish.

off whereas cells taken from rats treated additionally with blocking serum were washed off the dishes to the extent of 60%. It is noteworthy that cells from worm-infested rats adhered somewhat less firmly than controls, *i.e.* 20–30% of the cells were washed off. Thus, the ability of macrophages to cling to an appropriate

surface is diminished markedly by serum blocking factor, but their migration into the peritoneal cavity, as elicited by a variety of stimulants, is quite unaffected.

To determine whether locomotion of macrophages was affected by serum blocking factor, the mobility of otherwise untreated peptone-induced macrophages was compared with that of macrophages pretreated with serum of rats infested with the parasite as described before. When these cells were tested immediately after their incubation, no difference in locomotion was detected. However, when an interval of 2–4 hours was allowed to elapse between incubation and testing, a marked difference was observed constantly: cells cultured in the presence of 10% worm serum no longer showed any sign of locomotion whereas cells cultured in medium containing normal rat serum manifested normal motility (Fig. 2a, 2b).

Non-specificity of serum blocking factor

The elimination of Walker carcinosarcoma cells by peptone-induced, activated macrophages taken from inbred DA rats occurred in the presence or absence of serum obtained from inbred Lewis rats which had been infected with *N. brasiliensis* larvae 30 days earlier. These two rat strains differ in major histocompatibility antigens (Palm, 1971). Results represented in Table III show that the presence of serum from *N. brasiliensis*-infested Lewis rats largely interfered with the elimination of tumour cells by DA effector cells. In other experiments, mouse peritoneal macrophages harvested 5 days after i.p. injection of FCA were co-cultured with EATC in the additional presence or absence of serum from *N. brasiliensis*-infested rats. The results given in Table IV show that the rat serum blocking factor inhibits the elimination of tumour cells in the mouse system as well. Preincubation of serum from *N. brasiliensis*-infested rats with Walker carcinosarcoma cells and/or worms of the nematode *N. brasiliensis* or the trematodes

TABLE III.—*Blocking Serum Factor: Interference with the Elimination of Walker Carcinosarcoma Cells by DA Rat Macrophages*

Serum added	Percentage of tumour cells recovered after 5 hours of incubation
Normal DA rat	34 (±7)
Normal Lewis rat	32 (±11)
N. brasiliensis-infested Lewis rat	90 (±14)*

Each value represents the mean of 12 experiments.
* Significantly higher than controls (Student's *t*-test; $P < 0{\cdot}001$).

TABLE IV.—*Blocking Serum Factor: Interference with the Elimination of Ehrlich Ascites Tumour Cells by C3H Mouse Macrophages* in vitro

Serum added	Percentage of tumour cells recovered after 5 hours of incubation
Normal rat serum	51 (±9)
Normal mouse serum	48 (±12)
Serum of *N. brasiliensis*-infested rats	83 (±14)*

Each value represents the mean of 10 experiments.
* Significantly higher than controls (Student's *t*-test; $P < 0{\cdot}001$).

Fasciola hepatica or *Dicrocoelium dendriticum* all significantly diminished its capacity to block the elimination of Walker carcinosarcoma cells by activated rat macrophages *in vitro* (Table V).

TABLE V.—*Effect of Previous Mixing of Serum from* N. brasiliensis-*infested Rats with Various Tissues on its Blocking Capacity*

Pretreatment of blocking serum	Percentage of Walker carcinosarcoma cells recovered after 5 hours of incubation†
None	88 (±7)
Walker carcinosarcoma cells	48 (±11)*
N. brasiliensis (adult worms)	47 (±8)*
Fasciola hepatica (adult worms)	54 (±9)*
Dicrocoelium dendriticum (adult worms)	56 (±4)*
Controls (normal rat serum)	38 (±8)

*Significantly lower ($P < 0{\cdot}001$; Student's t-test) than with untreated serum from *N. brasiliensis*-infested rats.

† Each value represents the mean of 12 experiments. 10% serum was always present in the incubate.

DISCUSSION

Our earlier investigations had established that, in the present model system, prevention of tumour growth is a cell mediated phenomenon probably achieved by activated macrophages (Keller and Jones, 1971; Keller, 1971; Keller and Hess, 1972) whereas enhancement of tumour growth is due to a factor appearing in the serum of rats infected with the nematode, *N. brasiliensis* (Keller, Ogilvie and Simpson, 1971; Keller and Jones, 1971). It is accepted that macrophages may have a role in destructive processes with an immunological basis (Gorer, 1956) and that immune macrophages can eliminate target cells either by phagocytosis (Bennett, Old and Boyse, 1964; Rabinovitch, 1970; Chambers and Weiser, 1972) or by killing them by cell contact (Granger and Weiser, 1964; Evans and Alexander, 1970; Alexander and Evans, 1971). Our earlier results led us to the conclusion that non-specific phagocytosis is the mechanism involved in the elimination of tumour cells in the present system (Keller and Jones, 1971; Keller, 1972; Keller and Hess, 1972).

The present work shows that the blocking factor appearing in the serum of rats immune to *N. brasiliensis* (Keller, Ogilvie and Simpson, 1971) is able to enhance tumour growth in otherwise untreated rats and moreover fully reverses tumour inhibition occurring during the intestinal phase of a primary *N. brasiliensis* infection or after an intraperitoneal injection of peptone. Since tumour growth was effectively enhanced when the inoculated tumour cells had previously been in contact with serum of *N. brasiliensis*-infested rats it seems clear that the blocking factor may be absorbed on to tumour cells.

Earlier experiments on the effect of blocking factor on the process of elimination of tumour cells by a macrophage monolayer *in vitro* had indicated that prevention of its formation might be an important feature in maintenance of tumour immunity (Keller and Jones, 1971; Keller, 1972). The present illustra-

tions demonstrate that after only 2 to 4 hours' contact with the serum from parasite-infested rats, the activated macrophages showed all signs of inactivation: the cytoplasmic extensions disappear, the ruffled membrane movements cease, the cells become rounded up, lose their characteristic ability to adhere to the glass surface and become immobile. Thus, the blocking factor seems to inhibit the elimination of target cells by some disturbance of the metabolic functions of the macrophage (Cohn, 1970; Roos, 1970; Karnovsky, 1962) without affecting its viability.

Earlier observations in which blocking serum factor activity was shown to be associated with DEAE fractions containing only rat IgG globulin were interpreted that it may represent antibody (Keller and Jones, 1971). The present findings argue against its IgG identity for four principal reasons: (1) the rat serum blocking factor not only reverses the inhibition of tumour growth induced by a nematode infection in rats but also that induced by peptone, (2) the rat serum factor inhibits the *in vitro* elimination of tumour cells in a rat system differing in major histocompatibility antigens, (3) it is effective even in mouse macrophage/target cell system and (4) blocking activity is significantly diminished not only after mixing with Walker carcinosarcoma cells or *N. brasiliensis* adult worms but also after incubation with antigenically unrelated trematodes such as *Fasciola hepatica* and *Dicrocoelium dendriticum*. These findings are viewed as convincingly demonstrating that the effect is immunologically non-specific and that it is probably mediated by way of the macrophages.

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