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MACROPHAGES AND THE TUMOUR BEARING HOST

R. EVANS

From the Department of Tumour Immunology, Chester Beatty Research Institute, Laboratories at Clifton Avenue, Belmont, Sutton, Surrey

THE antimicrobial activity of macrophages *in vivo* or *in vitro* is frequently regarded as nonspecific. However, reports by Mackaness (1969) and Simon and Sheagren (1971) suggest that in their systems the bactericidal action is complex and probably involves an initial specific recognition of the immunizing antigen by the sensitized macrophage. Thereafter the macrophage becomes activated and is able to kill unrelated organisms.

This situation has its parallel in tumour immunity. We have demonstrated that macrophages recovered from suitably immunized hosts are activated by contact with the tumour cells used for immunization and thereafter will kill nonspecifically (Evans and Alexander, 1972*a*). However, without the specific recognition stage, the macrophages allow unrelated tumour cells to grow in their presence.

Our interest initially was to establish *in vitro* model systems whereby we could reflect changes occurring in the immunized host, and 2 systems were studied intensively. The first was a syngeneic combination in which mice were immunized with irradiated syngeneic tumour cells (Evans and Alexander, 1970). We demonstrated that syngeneic immune macrophages were cytotoxic only to the specific target cells. Cytotoxicity was expressed as growth inhibition since the macrophages induced a state of cytostasis, following which the target cells died and disintegrated. The second system was an allogeneic combination in which for example C57B1 mice were immunized with unirradiated DBA/2 SL2 lymphoma cells (den Otter, Evans and Alexander, 1972). The peritoneal macrophages when tested *in vitro* differed from those of the syngeneic combinations in that they were cytolytic inducing a rapid release of ^{51}Cr from the labelled lymphoma cells. As with the syngeneic, however, the alloimmune macrophages were quite specific in their recognition and lysis of the target cells, and could be activated only by the specific target cells. Furthermore, it was clearly demonstrated that macrophages and lymphocytes could interact, following which normal macrophages were armed *in vivo* or *in vitro* by contact with sensitized lymphocytes (Evans and Alexander, 1972*b*). These armed macrophages then recognized the antigen used to sensitize the host and this was demonstrated using tumour cells or bacterial antigens (Evans and Alexander, 1972*a*). Indeed, the available evidence would support the view that macrophage cytotoxicity is dependent on this initial co-operation, whether *in vivo* or *in vitro*.

The usefulness of these *in vitro* model systems was apparent when studying immune responses in tumour bearing animals, which is the subject of this report. By applying these techniques we have demonstrated that the tumour bearing host mounts an immune response, as measured by the appearance of sensitized macrophages at a distant site as well as in the actively growing tumour itself.

MATERIALS AND METHODS

Animals.—Pure-line DBA/2 or C57B1 mice 8–10 weeks of age, and Chester Beatty Hooded and Wistar rats 10–12 weeks of age were used for the various experiments.

Cells.—(a) DBA/2 lymphoma cells (SL2), maintained as an ascites tumour by weekly i.p. passage, were implanted subcutaneously

(s.c.) into DBA/2 mice, and at intervals thereafter the peritoneal macrophages were harvested and maintained as monolayers in Sterilin 3 cm plastic culture dishes as described previously (Evans and Alexander, 1970). At the same time spleens were removed and minced finely to prepare a suspension of cells which were washed × 3 by centrifugation and resuspension. The final suspension contained 5×10^6 spleen cells per ml of growth medium (Fischer's medium for leukaemic cells supplemented with 10% heat inactivated foetal bovine serum). (b) C57Bl lymphoma cells TLX9 were grown in culture and used as required. (c) C57Bl fibrosarcoma cells (FS6), serially passaged by s.c. implantation, were implanted s.c. and the procedure for preparation of macrophage cultures or spleen cell suspensions was the same as outlined in (a). FS6 cells were grown in culture and subcultivated at weekly intervals. (d) Chester Beatty Hooded rat fibrosarcoma cells (HSN and A), both passaged at regular intervals by s.c. implantation, were implanted s.c., and at intervals peritoneal macrophage cultures and spleen cell suspensions were prepared as above. The HSN was grown in culture and subcultivated weekly. For some experiments the G1 tissue culture line of fibrosarcoma cells, kindly supplied by Dr C. Dean was used. These were derived from a methylcholanthrene-induced tumour (MCI) and maintained in culture.

Method of assaying macrophage cytotoxicity

Following exposure of the target cells to the macrophages under test, 2 methods of assessing cytotoxicity were used: (a) lymphoma cells, SL2 and TLX9 grow in suspension, and may be counted by removing aliquots of cells at intervals and counting them in a haemacytometer; (b) fibrosarcoma cells adhere to and spread in culture vessels. By using the resistance of macrophages to detachment by trypsinization, adherent fibrosarcoma cells may be separated from macrophages by exposing cultures to 1 ml of 0·1% trypsin (Sigma 2X crystallized) for 30–45 min at 37°C. During this time tumour cells detached while macrophages were stimulated to spread out, as described in another system (Evans, 1973). The tumour cells could then be counted in a haemacytometer. Cytotoxicity involves an initial cytostasis during which the target cells fail to divide. This reaction is distinct from cytolysis which has been described for alloimmune macrophages (den Otter *et al.*, 1972).

Endotoxin and Poly I Poly C.—Preparations were described elsewhere (Alexander and Evans, 1971).

Fixation and staining.—Cultures were fixed in methanol and stained with Giemsa.

RESULTS

Growth inhibitory macrophages from tumour bearing hosts

(a) DBA/2 SL2 cells were implanted s.c. At 7 and 14 days peritoneal macrophage cytotoxicity was tested against SL2 and TLX9 (C57Bl) cells. Table I shows that at 7 days growth inhibition of SL2 cells was weak compared with 14 days which was much stronger. These macrophages did not inhibit growth of TLX9 cells at either time tested.

(b) Rat HSN fibrosarcoma pieces were implanted s.c. by trocar and 14 days later the cytotoxicity of the peritoneal macrophages was tested against a tissue culture line of HSN cells. The HSN cells were added at a concentration of 5×10^4 cells per 3 ml of growth medium to each culture dish. At least 4 dishes were counted at each sampling time (Fig. 1). It is seen that there was inhibition of growth over a

TABLE I.—*Growth Inhibition of SL2 Cells by Peritoneal Macrophages from Tumour Bearing Mice**

Macrophages tested after	Percentage growth inhibition at 48 hours	
	SL2	TLX9
7 days	29 ± 6	< 10%
14 days	71 ± 8	< 10%
Normal macrophages	< 10%	< 10%

* DBA/2 mice injected with 10^5 SL2 cells.

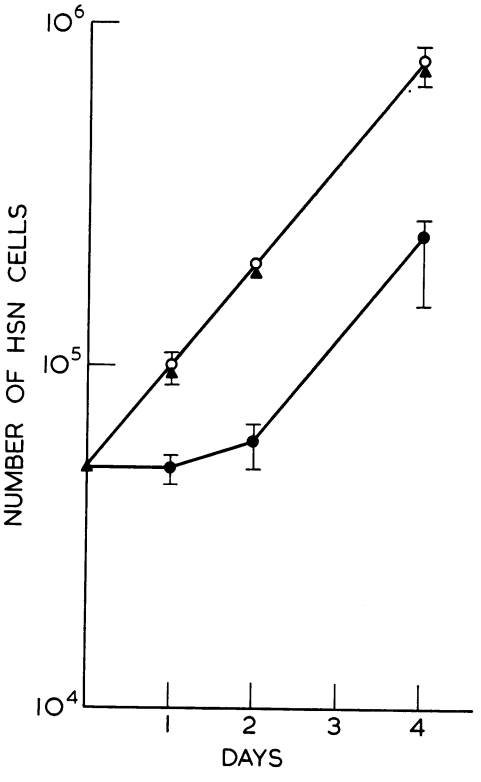

FIG. 1.—Growth inhibition of HSN tumour cells by peritoneal macrophages from HSN tumour bearing rats. ●—● peritoneal macroages from tumour bearing rats. ○—○ macrophages from normal rat. ▲—▲ no macrophages.

period of 48 hours but thereafter the fibrosarcoma cells grew at the normal rate. However, this reaction appeared to be dose dependent; 10^4 challenge cells failed to grow in these macrophage cultures. Rat GI cells were not inhibited in their growth.

Arming of normal macrophages by spleen cells

The spleens from DBA/2 mice implanted with SL2 cells were removed at 14 days and cell suspensions were added to cultures of normal macrophages. After 6 hours incubation the monolayers were washed thoroughly and challenged with SL2 or TLX9 cells. The results indicated that growth inhibition occurred and was specific for SL2 cells.

Similar experiments were carried out using spleens from mice implanted with FS6 fibrosarcoma and rats implanted with the HSN and MCI tumours to give positive evidence of arming and subsequent growth inhibition.

Macrophages in growing fibrosarcoma

Since it was evident that macrophages at a distant site, *i.e.* the peritoneal cavity, were specifically growth inhibitory to the target cells *in vitro*, it was of interest to ascertain whether cytotoxic macrophages reached the growing tumour, and whether they could be demonstrated to possess cytotoxic potential *in vitro*. As reported previously (Evans, 1972) it was shown that in a wide range of immunologically distinct fibrosarcomata in mice and rats, macrophages were present at varying concentrations but for a given tumour the concentration was fairly constant within limits, *e.g.*, rat tumours such as A and HSN and mouse FS6 had relatively high percentages of macrophages (40–60%), while the rat MC3 and mouse FS4 had low concentrations ($< 10\%$). To determine whether these macrophages expressed any cytotoxic activity towards target cells *in vitro*, tumour macrophage monolayers were prepared as described in detail elsewhere (Evans, 1973) and maintained in serum-free medium for 24–48 hours to eliminate background tumour cells which may have been carried over into the cultures during the initial preparation. The monolayers were then challenged with a standard dose of tumour cells (5×10^4 per culture in 3 ml of growth medium). The number of fibrosarcoma cells was assessed at intervals as described in Materials and Methods. A typical example (HSN) is given in Fig. 2, and Fig. 3–6 illustrate the sequence of events occurring during the cytotoxic response. Growth of the challenge tumour cells was arrested for approximately 48 hours, during which time macrophages rounded up and aggregated round the tumour cells (Fig. 3). This intense aggregation led to arborization of the tumour cells (Fig. 4), and finally to rounding up, detachment and death (Fig. 5). The

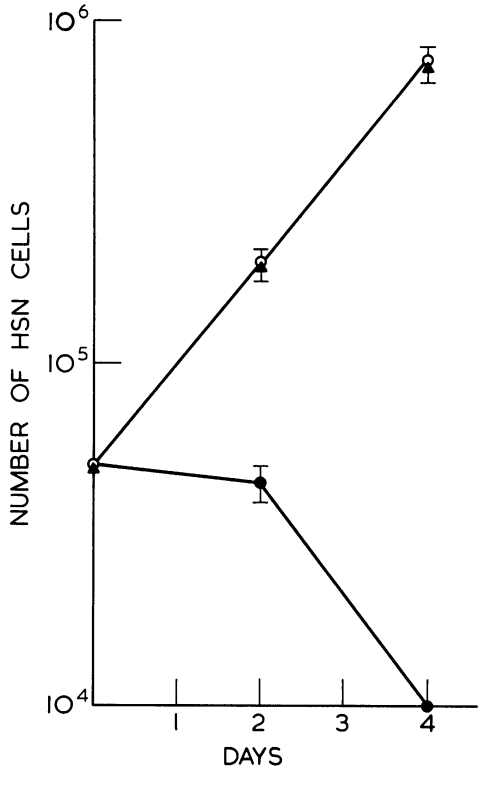

FIG. 2.—Growth inhibition of HSN tumour cells by HSN tumour macrophages. ●—● HSN tumour macrophages. ○—○ normal peritoneal macrophages. ▲—▲ no macrophages.

macrophages remained adherent to the detached cells, but during trypsinization they detached and were seen to re-settle and spread out on the culture surface (Fig. 6). Such macrophages were shown to have typical macrophage-monocyte-type nuclei and to be able to ingest opsonized sheep red blood cells. By 5–7 days after challenge the macrophage cultures were relatively free of tumour cells compared with control cultures of either fibrosarcoma cells alone or tumour cell monolayers of normal peritoneal macrophages.

Specificity of cytotoxicity

Results regarding specificity of the growth inhibition were not consistent. For example, on some occasions HSN tumour cells failed to grow on A tumour macrophages whereas in other experiments growth was normal. Similarly, the GI tumour cells usually grew normally on HSN and A tumour macrophage monolayers but from time to time growth inhibition was marked. These inconsistencies may be related to tumour size as well as the length of time the tumour has been growing. Further experiments are in progress to explore these problems.

Enhancement of macrophage cytotoxicity

As demonstrated previously (Alexander and Evans, 1971), peritoneal macrophages pre-treated with endotoxin, viral double-stranded RNA and synthetic Poly I Poly C were activated to kill a range of tumour cells in a nonspecific manner. To test whether tumour-associated macrophage cytotoxicity could be enhanced by such treatment, rat HSN and A, and mouse FSI and FS6 tumour macrophages were treated at different times with endotoxin or Poly I Poly C (50 μg per culture) for 4 hours, washed and then challenged with 5×10^4 tumour cells. A typical example is shown in Table II in which treated and untreated HSN and A tumour macrophages were challenged with the unrelated GI fibrosarcoma cells. It is seen that on the endotoxin or Poly I Poly C-treated cultures the GI cells failed to grow and indeed death of cells was apparent. In the untreated macrophage cultures, or in dishes devoid of macrophages, GI cells grew normally. Neither endotoxin nor Poly I Poly C affected growth of GI cells at the concentration used. Similar results were obtained using the FS1 and FS6 tumour macrophages.

DISCUSSION

The above *in vitro* data demonstrate that in the tumour bearing host, growth inhibitory macrophages are found in the peritoneal cavity and in the actively growing tumour itself. Furthermore, the spleen cells from such tumour bearing animals render normal macrophages cyto-

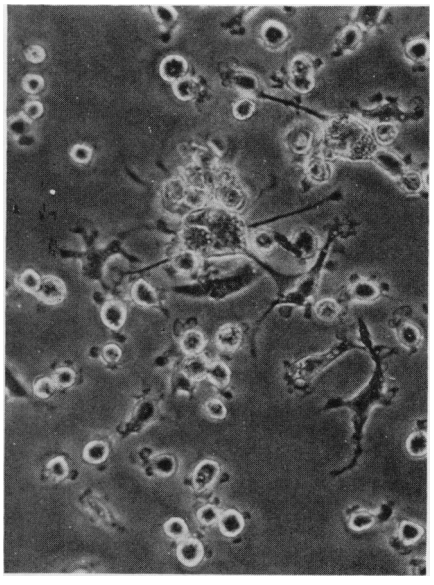

FIG. 3.—Rat HSN tumour macrophages showing signs of rounding up 24 hours after challenge with HSN tumour cells. Phase contrast × 192.

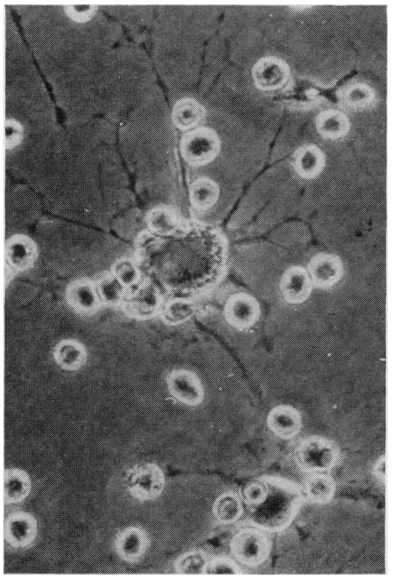

FIG. 4.—Rat HSN tumour macrophages aggregated round an arborized HSN tumour cell. Phase contrast × 296.

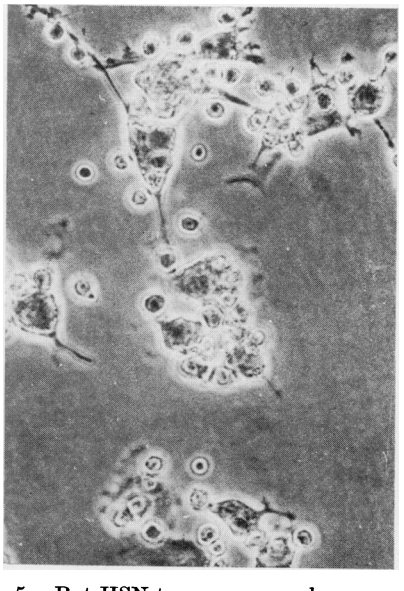

FIG. 5.—Rat HSN tumour macrophages aggregated round HSN tumour cells 4 days after challenge of macrophage monolayer. Phase contrast × 176.

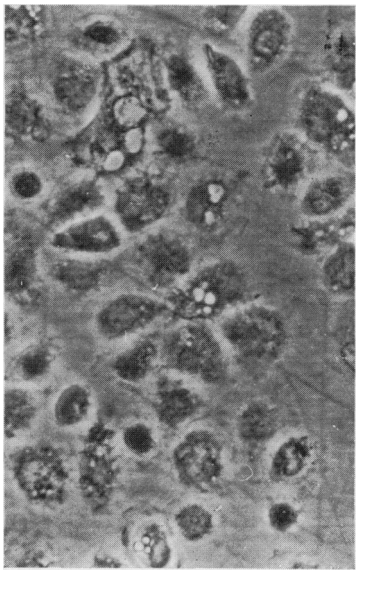

FIG. 6.—Rat HSN tumour macrophage monolayer after trypsinization of the mixed culture of tumour cells and macrophages for 45 min at 37°. The macrophages have re-adhered and spread out. No tumour cells are visible. Phase contrast × 270.

TABLE II.—*Tumour-macrophage Cytotoxicity after Treatment with Endotoxin or Poly I Poly C*

Treatment	Number of GI* cells after 48 hours on macrophage monolayers from	
	HSN tumour	A tumour
Endotoxin	$0{\cdot}2\times10^5\pm0{\cdot}2$	$0{\cdot}5\times10^4\pm0{\cdot}1$
Poly I Poly C	$0{\cdot}4\times10^5\pm0{\cdot}1$	$0{\cdot}1\times10^5\pm0{\cdot}1$
None	$1{\cdot}9\times10^5\pm0{\cdot}2$	$2{\cdot}0\times10^5\pm0{\cdot}2$
GI cells alone ± endotoxin or Poly I Poly C	$2{\cdot}1\times10^5\pm0{\cdot}1$	$2{\cdot}2\times10^5\pm0{\cdot}1$

* GI cells seeded at a concentration of $0{\cdot}5\times10^5$ in 3 ml of growth medium.

toxic to the specific target cells. These results indicate that the host mounts an immune response mediated against the tumour. This response, however, appears to be fairly weak as measured by the *in vitro* test systems and by concomitant immunity studies (Alexander, Evans and Mikulska, 1972), in which the number of grafted cells the tumour bearing host was able to reject was low compared with numbers rejected by suitably immunized syngeneic hosts. Since the tumour ultimately kills the host, it is apparent that the immune responses fail and that cell mediated killing is not effective. What relationship is there between *in vitro* cytotoxicity and the events taking place *in vivo*?

There are probably many different though perhaps inter-related reasons why the cytotoxic macrophages in the tumour fail to bring about rejection. One reason must relate to the validity of the *in vitro* test system, whether in fact macrophages cytotoxic *in vitro* are cytotoxic *in situ*. Simply on a cell-to-cell basis one might not expect a ratio of 2 macrophages to 1 tumour cell (this is the highest ratio found in the rat A tumour) to have much effect on tumour growth when *in vitro* at least 20–40 macrophages to 1 target cell was required to demonstrate cytotoxicity. It is not known why there is a consistent proportion of macrophages at all stages of the tumour growth, but clearly a limiting factor in deciding how many macrophages infiltrate the growing tumour at any particular time must be related to the capacity of the bone marrow to meet the demands. Without the knowledge of the turnover rate of macrophages in the tumour mass, we do not know the kinetics of proliferation or infiltration of macrophages or monocytes. As far as we are aware, division of macrophages within the growing tumour does not occur, and this implies that there must be a massive infiltration to keep pace with the dividing tumour cells. Such are the demands on the sequestration of macrophages in the tumour that delayed hypersensitivity reactions against defined antigens are negligible or not measurable (S. Eccles, personal communication). Even so, the reactions can be restored to a large extent by injection of normal peritoneal exudates, but not by thoracic duct lymphocytes. Whether this consistent level of macrophages is a direct measure of the immune response mounted by the host requires further study. Another reason for the continued growth of the tumour when cytotoxic cells can be demonstrated *in vitro* is the possible effect blocking factors may have on macrophages. If such factors bind to macrophages, as they do to sensitized lymphocytes (Currie and Basham, 1972), the macrophage-mediated cytotoxicity would be ablated *in situ*. As demonstrated by Currie and Basham (1972) and Currie and Gage (personal communication), lymphocytes from tumour bearing patients or rats required extensive washing to mediate their cytotoxicity *in vitro*. Macrophage-bound factors could well be removed during the procedure involved in preparation of monolayers (*e.g.* prolonged trypsinization.

Whatever the reasons for the inadequacy of cell mediated responses in the tumour bearing host, the fact remains that the tumour grows and frequently metastasizes. Whether cytotoxic macrophages exert any restricting effect on overall tumour growth rate, or prevent or retard metastatic spread, remains to be elucidated. Preliminary observations (Rudenstam and Evans, unpublished data) suggest a circumstantial relationship at least between high macrophage content of particular rat tumours and the low rate of metastatic spread to the lungs, the converse applying to tumours with low macrophage content. Experiments are currently under way to extend these findings and to determine whether tumour growth and macrophage infiltration are affected after treatment of animals with immunosuppressants or corticosteroids, which affect peripheral blood macrophage levels (Thompson and van Furth, 1970).

At present the data are too few to be able to propose a precise role for macrophages found in actively growing tumours. While one might suggest a direct involvement for peritoneal macrophages following rejection of an i.p. graft, involvement of macrophages associated with the tumour mass in the slowing of growth rate or of metastatic spread has not been established. Various *in vitro* experiments suggest how tumour macrophages might be exploited, for example, treatment of these macrophages with endotoxin or dsRNA rendered them cytotoxic in a nonspecific manner to fibrosarcoma cells. In this light, the recent results from this laboratory (Parr, Wheeler and Alexander, 1973) are interesting in that treatment of tumour bearing mice with either compound resulted in regression of tumours under well defined conditions. It is tempting to speculate that macrophages within or outside the tumour mass might be involved in the rejection. However, the sequence of events is likely to be highly complex, involving blood vessel damage amongst other reactions. Nevertheless, it should be possible to investigate reactions such as this at the cellular level since regression occurs very rapidly after treatment, and to determine the extent, if any, of involvement of macrophages.

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