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Inhibition of cytokine production by a tumour cell product

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Summary. Supernatants from cultured mouse and human tumour cells, but not mouse or guinea-pig fibroblasts, inhibited the production of a lymphokine, macrophage chemotactic factor, by PHA-stimulated mouse spleen cells. The supernatants affected spleen cells from old, but not young, mice. They were most active if added at the start of the spleen cell culture and did not act by binding phytohaemagglutinin (PHA). The active material had an approximate molecular weight, on membrane filtration, of 1000–10,000 and could be bound to and eluted from Con A-Sepharose. Tumour supernatant factor(s) of similar molecular weight inhibited the production of interleukin 1 (lymphocyte activating factor) in response to lipopolysaccharide by stimulated thioglycollate-induced peritoneal exudate macrophages, but not by *Corynebacterium parvum*-activated macrophages. Similar tumour-produced material has been found to inhibit the early phase of delayed-type hypersensitivity reactions in older mice. It is suggested that this effect is due, at least in part, to inhibition of interleukin 1 production leading to inhibition of lymphokine production.

INTRODUCTION

There is much evidence that macrophages are important effectors of natural and acquired resistance to many tumours. Such evidence includes cytotoxicity of some macrophages to target tumour cells *in vitro*, the promotion of tumour growth by anti-macrophage agents *in vivo* and the apparently direct suppression of growth of some tumours by stimulated macrophages (for reviews see: Evans & Alexander, 1976; James, McBride & Stuart, 1977; Hibbs, Chapman & Weinberg, 1978; Nelson, Hopper & Nelson, 1978; Keller, 1980). The effectiveness of macrophage-mediated defences may, however, be vitiated by the ability of tumours to depress macrophage function and inflammatory reactions. Tumour-bearing animals have shown an impaired capacity to mobilize macrophages to the sites of non-specific inflammation (Eccles & Alexander, 1974; Normann & Sorkin, 1976) and to delayed-type hypersensitivity (DTH) reactions (Eccles & Alexander, 1974; Wells, Cain, Wells & Bozalis, 1974; Jessup, Cohen, Tomaszewski & Felix, 1976). Soluble products of tumour cells may be responsible for these effects, as they have been found to inhibit macrophage mobility *in vitro* and inflammatory reactions *in vivo*: they may also promote tumour growth *in vitro* (Fauve, Hevin, Jacob, Gaillard & Jacob, 1974; Pike & Snyderman, 1976; reviewed by Nelson, Nelson, Farram & Inoue, 1981).

Studies in this laboratory showed that supernatants from cultures of a wide variety of tumours could depress DTH reactions in mice and inhibit macrophage migration (Nelson & Nelson, 1978). Inhibition of DTH reactions did not appear to be due to a direct effect on the mobility of mononuclear phagocytes, since DTH was depressed only in mice more than 4 months old, whereas macrophages from mice of any

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age were susceptible, and macrophage migration was inhibited by material of apparent mol. wt less than 1000, whereas DTH was inhibited by two factors of apparent mol. wt 1000–10,000 and greater than 10,000 (Nelson & Nelson, 1980; Nelson *et al.*, 1981). It seemed possible that inhibition of monocytopoiesis might be responsible but this, too, was brought about by low molecular weight material lacking an inhibitory effect on DTH (Farram, Nelson & Nelson, 1981). Although tumour supernatants did not selectively or dramatically inhibit lymphoproliferative responses *in vitro* (Nelson & Nelson, 1978) it seemed possible that they might inhibit lymphokine production. The effects of tumour supernatants and fractions thereof on the production of a lymphokine, lymphocyte-derived macrophage chemotactic factor (LDCF) were therefore examined, this lymphokine being selected both for convenience and for its obvious relevance to DTH reactions. The effects on the monokine interleukin 1 were also examined because of its role as a lymphocyte activating factor.

MATERIALS AND METHODS

Animals

CBA/J mice were either obtained from The Jackson Laboratory (Bar Harbor, Maine) or bred at the Gore Hill Research Laboratories (Sydney) from pedigreed stock from the Jackson Laboratory. All mice used were older than 17 weeks unless otherwise stated.

Media

The following media were used: Dulbecco's modified Eagles' medium (DME) and RPMI 1640 (both from Gibco, Grand Island, New York) containing penicillin (100 u./ml) and streptomycin (100 μg/ml); foetal calf serum (FCS) from Flow Laboratories (Australia) was inactivated at 56° for 30 min.

Cells and cultures

A-2 (a 3-methylcholanthrene-induced fibroscarcoma of A/J mice), SCS-3 (a spindle cell sarcoma that arose spontaneously in a CBA/J mouse), MM200 (human malignant melanoma) and CBA/J mouse and guinea-pig fibroblast cultures were passaged, stored and maintained as previously described (Kearney & Nelson, 1973; Nelson & Nelson, 1978, 1980; Farram & Nelson, 1980). T470, a human breast cancer cell line was obtained from Professor I. F. C. Mackenzie (Department of Pathology, University of Melbourne).

Resident peritoneal cells were obtained from mice, killed by cervical dislocation, by lavage with 4 ml of DME containing 10 u./ml preservative-free heparin (Commonwealth Serum Laboratories, Melbourne). Stimulated peritoneal macrophages were similarly obtained from mice that had received intraperitoneal (i.p.) injections of 2 ml thioglycollate medium (BBL, Microbiology Systems, Md) 4–7 days previously or 0·28 mg of *Corynebacterium parvum* (UL 01, Wellcome) 7–14 days previously. The cell suspensions, which were washed twice, contained approximately 80% macrophages by neutral red stain (Cohn & Wiener, 1963). All incubations were at 37° in a moist atmosphere of 5% CO_2 in air.

Cell supernatants and fractions

Supernatants were obtained from tumour and fibroblast cultures incubated for 24 hr in serum-free medium and were processed as previously described (Farram *et al.*, 1981) by passage through Amicon 'Diaflo' membranes. Fractions of the following approximate mol. wt were obtained: >10,000 (G10), 1000–10,000 (L10) and <1000 (L1). These fractions together with whole unfractionated supernatants were used in the experiments described. They were added to cultures at the final concentrations shown in the results.

Purification procedures

Confluent normal or tumour cell cultures were incubated for 24 hr with 15 ml of serum-free, antibiotic-free and phenol red-free medium (MEMP, Auto-Pow, Flow Laboratories, N.S.W., Australia). The cell supernatants were harvested, pooled and stored at 4°. Batches of 200 ml of cell supernatants were filtered through a 'Diaflo' PM10 membrane (cut-off approximately 10,000) and the filtrate through a UM2 membrane (cut-off approximately 1000). Material retained by the UM2 membrane was washed with saline and applied to a Con A-Sepharose column, 1·77 $cm^2 \times 27$ cm, equilibrated in saline. Bound material was eluted with 0·1 M α-methyl mannoside in 1 M NaCl. The eluate was dialysed against 3 × 1 litre of water using dialysis tubing of approximate cut-off 3500 (Thomas, Philadelphia, Pa). The retentate was lyophilized and stored at −20°. Before use, the material was dissolved in 1 ml of water, and aliquots diluted in appropriate volumes of DME for assay.

Chemotaxis

The *in vitro* chemotactic response of mouse

macrophages to lymphocyte-derived chemotactic factor (LDCF) was assayed according to the method of Meltzer (1976) with some modifications. LDCF was produced by incubating mouse spleen cells (5×10^6/ml) with or without PHA-P (Difco Laboratories, 100 μg/ml) in Linbro 24-well tissue culture plates (Flow Laboratories, catalogue number 76-033-05) in duplicate 1 ml volumes of DME with 10% FCS. The cultures were incubated for 3 days, supernatants were harvested, diluted 1 in 3 with the same medium and assayed for LDCF. For control cultures, PHA-P was added immediately before harvest. In experiments where cell culture supernatants and fractions were tested for their effects on LDCF production, the fractions were added at the start of cultures except in kinetic studies. Initially, a 32-well blind well chamber (designed by Dr K. Hopper) was used. The assembly consisted of an upper and lower plate (200 μl per well volume) separated by O-rings and Nuclepore filter discs (5×13 μm, catalogue number 341-1001, Bio-Rad Labs, Calif.). In later experiments a 48-well microchemotaxis chamber kit (Neuroprobe Inc., Bethesda, Md) was used. The method has been described in detail (Falk, Goodwin & Leonard, 1980). The two types of chamber gave similar results. For the microchambers, 25 μl of LDCF-containing supernatants were placed in triplicate wells. The top wells were filled with 0·05 ml of peritoneal exudate cells (2×10^6/ml in DME with 10% FCS) from thioglycollate-injected CBA/J mice. After 3 hr incubation the filter sheets (5 μm, $7{\cdot}6 \times 2{\cdot}5$ cm) were removed, fixed, stained with Wright's stain and mounted on glass slides. Macrophages which had migrated through the filter were counted with a forty times objective and eyepiece grid. Five grid fields were counted per well and the mean of triplicate wells determined for each supernatant assayed. Results are expressed as the number of macrophages migrated per 5 grid fields in test wells minus the number of macrophages migrated per 5 grid fields in control wells (containing supernatants from spleen cell cultures to which PHA-P had been added at the end of incubation).

Lymphocyte-activating factor (interleukin 1)

Interleukin 1 (IL1) was produced by lipopolysaccharide (LPS, endotoxin) stimulated macrophages from thioglycollate-injected mice. Peritoneal exudate cells were seeded into 35 mm plastic petri dishes (Falcon Plastics, 5×10^6/ml in RPMI 1640 with 10% FCS). After a 3 hr incubation the monolayers were washed three times and reincubated in the same medium containing *Escherichia coli* W LPS, 10 μg/ml (Difco Laboratories) in the presence or absence of tumour supernatant factors. After 24 hr the supernatants were collected, dialysed against RPMI 1640 for 24 hr at 4° with two changes of medium and stored at −70° until used. IL1 activity was tested in thymocyte cultures. Thymocytes were obtained from 4–6 week old mice. The cultures were set up in quadruplicate in 96-well Linbro tissue culture plates (Flow Labs, catalogue number 76-003-05) with 1×10^6 cells/well in RPMI 1640 with 10% FCS and 10^{-5} M 2-mercaptoethanol. IL1 supernatants (prepared in the presence or absence of tumour fractions) were added at various concentrations. Cultures were incubated in the presence of PHA-P (1 μg/well) for 72 hr and then pulsed overnight with [Me-^{3}H]-thymidine (6·7 Ci/mmol, New England Nuclear, Boston, Mass., 0·3 μCi/well). Cultures were harvested on a Skatron cell harvester and counted in a Packard liquid scintillation counter. Thymidine incorporation is expressed as the mean counts per min/culture from quadruplicate cultures.

Statistical analysis

Student's *t* test was used.

Lactic dehydrogenase elevating virus

Every 3–6 months, tumour-bearing mice were bled and lactic dehydrogenase activity in serum was measured by means of commercial kits (Curtin-Matheson Scientific, Houston, Tx., or Ultrazyme, Harleco, Australia). To date, no tumour-bearing mice have shown the marked elevation of enzyme activity characteristic of LDH virus infection (Riley, 1968).

RESULTS

The effect of tumour and cell culture supernatants on macrophage chemotaxis

Direct migration. Preliminary experiments carried out established the optimal conditions for producing and detecting LDCF in the chemotaxis assay. A one in three dilution of LDCF supernatant was selected from the middle of the dose–response curve and this concentration also gave a suitable number of migrated macrophages for counting (approximately 100 cells per 5 grid fields).

Firstly, the direct effect of tumour and cell culture supernatant factors on the response of macrophages to LDCF was examined (Table 1). For SCS-3, A-2,

Table 1. The effect of tumour and fibroblast culture supernatants on migration of macrophages in the chemotaxis assay

Tumour or cell culture	Concentration (%)	Number of macrophages § migrating in the presence of culture supernatants or fractions				
		Nil	Whole	G10	L10	L1
SCS-3	—	113±8				
	20		54±6‡	(102±2)	95±3*	41±6‡
	10		64±6†	(100±3)	(98±4)	43±3‡
	5		70±3†	(112±8)	(98±8)	69±4†
A-2	—	107±5				
	20		76±3‡	(100±3)	94±0*	71±6‡
	10		87±4†	(102±3)	95±2*	78±6†
	5		90±3*	(102±0)	(98±3)	93±2*
MM200	—	103±7				
	20		71±4‡	(90±9)	90±4*	77±9†
	10		85±7*	(100±3)	(102±5)	91±3*
	5		91±5*	(110±0)	(97±4)	95±1*
T470	—	106±6				
	20		43±4‡	(92±3)	87±3*	47±2‡
	10		59±3‡	(99±1)	89±10*	66±6†
	5		80±0*	(98±1)	(99±11)	83±8†
Guinea-pig fibroblast	—	99±9				
	20		(103±9)			(99±9)
	10		(112±5)			(105±3)
CBA/J fibroblast	—	78±2				
	20		108±10*	(77±2)	86±2*	(76±1)
	10		(78±7)	(77±0)	(83±2)	(70±11)
	5		(79±9)		(73±2)	(79±5)

Supernatant fractions were mixed with LDCF (final concentration 1 in 3) produced as described in Materials and Methods at the concentrations shown and placed in the lower wells of chemotaxis chambers.

* $0.05 \geq P \geq 0.01$.

† $0.01 \geq P \geq 0.005$.

‡ $P < 0.005$.

§ Results are expressed as mean of triplicates ± SEM.

Figures in parentheses are not significantly different from controls ($P > 0.05$).

MM200 and T470 tumours, the whole unseparated fractions all showed significant inhibition of macrophage migration at all three concentrations tested. When the supernatants were fractionated, the greatest inhibitory activity was concentrated in the L1 fraction. Some inhibition (not more than 20%) was also found in the L10 fraction at 20% and 10% concentration and this could be due to incomplete separation of factors. There was no activity in G10 fractions from any of the tumours. On the other hand, comparable fractions from guinea-pig and CBA/J fibroblast fractions had no inhibitory activity. The tumour supernatants themselves were tested for chemotactic activity and were found to have none. They were not toxic to peritoneal exudate cells, as shown by normal survival and morphology of cells cultured with tumour supernatants for 3–24 hr.

Production of LDCF

Table 2 shows the effect of culture supernatants on production of LDCF, reflected in the number of

Table 2. Effects of tumour and fibroblast culture supernatants on the production of LDCF

Tumour or cell culture	Concentration (%)	LDCF activity, as number of macrophages migrated§ in the presence of supernatants from spleen cells cultured with PHA and culture supernatants or fractions				
		Nil	Whole	G10	L10	L1
SCS-3	—	73±4				
	20		41±4‡	84±3*	38±3	(73±4)
	10		45±3‡	(70±3)	42±8‡	80±2*
	5		(72±3)	(77±5)	46±5†	(77±7)
A-2	—	99±3				
	20		69±2‡	86±5*	53±4‡	(102±5)
	10		67±7‡	(91±3)	74±2‡	(98±3)
	5		86±5*	(93±2)	88±4*	109±1†
MM200	—	110±5				
	20		48±5‡	91±11*	40±1‡	91±9*
	10		57±5‡	(99±2)	52±6‡	(100±8)
	5		100±9*	(101±1)	84±4†	(99±7)
T470	—	88±8				
	20		38±3‡	70±2*	43±5‡	69±7*
	10		52±4†	(80±4)	54±4‡	(74±2)
	5		65±3†	(77±6)	64±5*	(91±6)
Guinea-pig fibroblast	—	97±12				
	20		119±1*		115±12*	
	10		(112±2)		(96±14)	
	5		(100±3)		(95±9)	
CBA/J fibroblast	—	74±2				
	20		(69±4)		(69±3)	
	10		(64±8)		(69±4)	
	5		(66±8)		(70±3)	

*†‡§ As for Table 1.
Tumour and fibroblast culture supernatants were added to spleen cell cultures at the concentration shown.

macrophages migrating in the chemotaxis assay. Whole supernatant and the L10 fraction from tumour cells (SCS-3, A-2, MM200 and T470) depressed LDCF production by 50% or more at 20% concentration. Slight inhibition or enhancement (not more than 20%) was obtained with G10 and L1 fractions. Supernatants from cultures of guinea-pig and CBA/J fibroblasts did not depress LDCF production.

Kinetics of inhibition of LDCF production

To examine the kinetics of inhibition of LDCF production L10 fraction from SCS-3 tumour was added at the start of culture (day 0), on day 1 or on day 2. On day 3 all cultures were harvested and LDCF activity was tested. In Fig. 1 it can be seen that maximum inhibition (approximately 66%) of LDCF production by the L10 fraction was obtained when it was added at the start of culture. Much less inhibition (30% or less) was obtained when the L10 fraction was added after day 0.

The influence of age on inhibition of LDCF production by L10 tumour fraction

As previously reported (Nelson & Nelson, 1980) depression of DTH by tumour supernatants was detectable only in mice more than 4 months old.

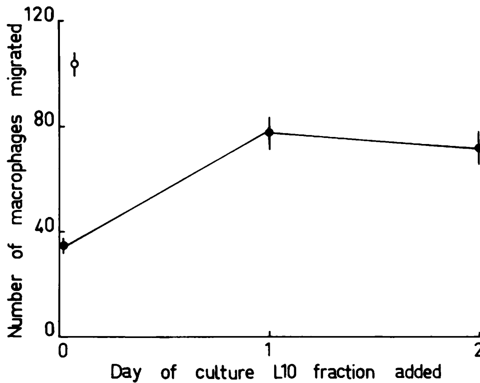

Figure 1. Time course of inhibition by SCS-3 tumour fraction L10 of LDCF production. L10 was added to cultures on days shown (20% concentration) and all cultures were harvested on day 3 (●——●). LDCF produced in the absence of L10 fraction (—○—). Points represent the mean ± SEM of total macrophages migrating in five grid fields from triplicate cultures.

Younger mice were not susceptible. The effect of SCS-3 tumour culture supernatants on spleen cell cultures from old and young mice was therefore examined. The results in Table 3 show clearly that only cells from older mice were susceptible to inhibition of LDCF production by whole and L10 fraction of the supernatant.

Table 3. Effect of SCS-3 tumour supernatant on LDCF production by spleen cells from old and young mice

	Inhibition (%) of macrophage chemotactic activity*			
	Experiment 1		Experiment 2	
Tumour fraction (%)	Old mice	Young mice	Old mice	Young mice
Whole (20)	35	(7)	36	(7)
(10)	18	(−6)	29	0
L10 (20)	47	(5)	57	(13)
(10)	35	(−6)	36	(7)

* Inhibition (%) was calculated by comparing LDCF activity (as macrophages migrating) of cultures incubated in the presence of tumour supernatant or L10 fraction with that of cultures incubated without tumour supernatant or L10 fraction.

Figures in parenthesis are not significantly different from control ($P > 0{\cdot}05$). Others are significantly different ($P < 0{\cdot}01$). Young mice were 6–8 weeks old and old mice approximately 12 months old.

Purification of L10 fraction from tumour and cell cultures

Purification procedures of the L10 fractions were performed as described in Materials and Methods. The partially purified fractions were then tested in LDCF cultures as before. Results in Table 4 show that most of the inhibitory material from A-2 and SCS-3 tumours was retained in the fraction that bound to Con A-Sepharose columns (46% and 32% inhibition, respectively). The unbound fraction from A-2 tumours had no activity. Freeze drying and reconstitution of the material also did not alter its activity. Control fibroblast fractions treated in the same way were not inhibitory in LDCF cultures (−3% inhibition).

Mechanism of inhibition of L10 tumour fraction on LDCF production

Experiments were performed to determine whether suppression of LDCF production by the tumour inhibitor was due to depletion of PHA by binding to the L10 fraction. L10 fraction was preincubated with PHA before addition of spleen cells. The data in Table 5 indicate that the depression of LDCF production was not due to binding of PHA to the L10 fraction as inhibition was if anything greater than in cultures not preincubated. In the absence of freshly added PHA depression of LDCF production could have been interpreted as due either to depletion of PHA by binding to the L10 fraction or to direct inhibition. The addition of fresh PHA ensured that depression of LDCF production was not due to binding of PHA. Conversely, if the factor had bound to PHA during preincubation it should not have depressed LDCF production in the presence of fresh PHA.

It was thought that the tumour factor might act on lymphocytes producing LDCF or on macrophages producing IL1 in the early stages of lymphocyte activation. Initially experiments were done with L10 and macrophage-depleted spleen cells in the presence of IL1. There was, however, insufficient reduction of LDCF production (perhaps due to inadequate macrophage depletion) and restoration by IL1 for satisfactory interpretation. Instead, the action of the tumour inhibitor was examined indirectly, by studying its effects on IL1 production by LPS-stimulated macrophages. Tumour supernatants inhibited IL1 production and in Fig. 2 it can be seen that most of the inhibitory activity was associated with the L10 fraction. G10 and L1 fractions had much less activity. The

Table 4. The effect of partly purified tumour and cell culture supernatant fractions on LDCF production

Tumour or cell culture	Fraction	Number of macrophages migrating	Inhibition (%) of‡ macrophage chemotactic activity
—	—	78±1	
A-2	Whole, unseparated	33±1*	58
	Not bound, L10	78±1†	0
	Bound, L10	42±6*	46
—	—	84±3	
SCS-3	Bound, L10	57±5*	32
CBA/J fibroblast	Bound, L10	87±7†	−3

* $P<0·005$.
† $P>0·05$.
‡ As for Table 3.
Bound and unbound fractions from Con A-Sepharose columns were eluted and treated as described in Materials and Methods. They were then diluted to the original concentration and tested in LDCF cultures at 20% concentration. SCS-3 and fibroblast bound fractions were further treated by freeze drying, reconstitution and used as above.

Table 5. The effect of preincubation of SCS-3 tumour fraction L10 with PHA

Preincubation	L10 fraction	LDCF activity, as number of macrophages migrating*
—	—	80±12
—	+	42±3†
+	+	26±3†

L10 tumour fraction was preincubated with PHA (100 μg/ml) at 20% concentration overnight. The next day spleen cells (5×10^6/ml) and fresh PHA (100 μg/ml) were added and cultures incubated for 3 days.
* As for Table 1.
† $P<0·005$.

effect of the tumour fractions on IL1 activation of thymocytes was also examined. Only the G10 fraction need be considered as factors of mol. wt less than 10,000 would have been removed during dialysis of IL1 supernatants. However, as the G10 fraction caused only slight inhibition of IL1 production (approximately 20%, Fig. 2) its role in the IL1 assay seems unimportant. Moreover, addition of the G10 fraction directly to the IL1 assay did not suppress thymocyte activation significantly.

The effect of the L10 fraction on IL1 production was determined in greater detail. Using three concentrations (10%, 20% and 30%) it was found to inhibit IL1 production in a dose-related manner (Fig. 3).

To examine the susceptibility of activated macrophages the L10 fraction from SCS-3 tumour supernatant was also tested on *C. parvum* elicited macrophages. Table 6 shows that they were resistant to inhibition of IL1 production.

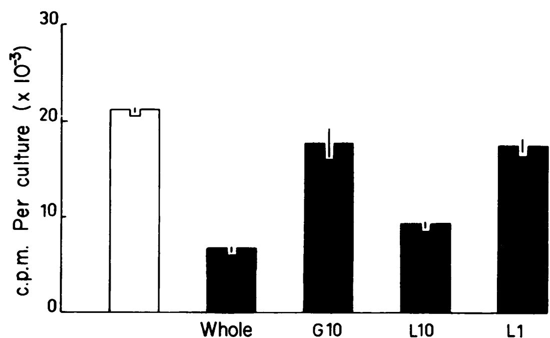

Figure 2. The effect of SCS-3 tumour factors on the production of IL1 by LPS-stimulated thioglycollate-induced macrophages. Macrophage monolayers were incubated with LPS and medium (□) or tumour factors at 20% final concentration (■). Thymocyte activation by PHA was estimated with the macrophage supernatants (IL1) at 15% concentration.

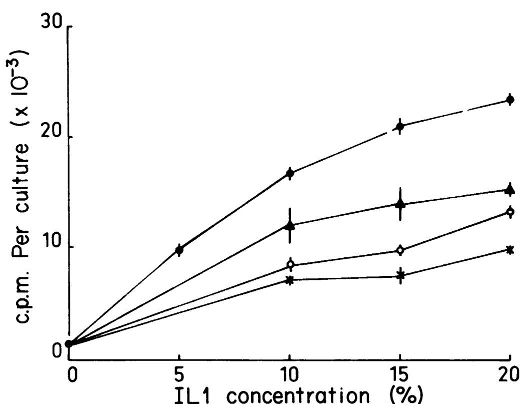

Figure 3. The effect of different concentrations of L10 fraction from SCS-3 tumour supernatant on the production of IL1 by LPS-stimulated thioglycollate-induced macrophages. The macrophages were incubated with LPS and medium (●——●) or L10 fraction at 10% (▲——▲), 20% (○——○) and 30% (*——*) concentration. Thymocyte activation by PHA was measured in the presence of macrophage supernatants (IL1) at different concentrations.

Table 6. The effect of SCS-3 tumour supernatants on the production of IL1 by *C. parvum* stimulated macrophages

SCS-3 tumour fractions	IL1 activity*	Inhibition (%) of IL1 production
None	6556±678	
Whole supernatant	7816±993	−16
L10	6852±143	4

* Tritiated thymidine incorporation into PHA stimulated thymocyte cultures (mean c.p.m. ±SEM of quadruplicate cultures) in the presence of IL1. Incorporation in the absence of IL1: 496±48 c.p.m.

DISCUSSION

The results of these experiments support the hypothesis that tumour-produced factors may depress DTH reactions in part by inhibiting the production of a lymphokine chemotactic for macrophages. It was thought that testing this hypothesis might present technical difficulties because of the capacity of fresh tumour culture supernatants to inhibit macrophage migration directly (Nelson & Nelson, 1978). This effect was again seen and was almost entirely associated with the L1 fraction (Table 1). Reduced migration of macrophages in the presence of supernatants from PHA-stimulated spleen cells cultured with tumour supernatants seems to be due to reduced LDCF production rather than persistence of directly inhibitory material because (i) the reduction was pronounced with the L10 fraction which had no direct effect on macrophage migration; (ii) there was little or no reduction with the L1 fraction; (iii) reduction was in many cases greater when the spleen cell cultures contained supernatant or L10 fraction at 5% concentration (further diluted 1 in 3 in the assay of LDCF) than when tumour supernatants or L1 fractions were tested at 10% or 20% concentration for a direct inhibitory effect on migration in response to preformed LDCF. The loss of the direct inhibitory effect of the L1 fractions may have been due partly to dilution and partly to breakdown during the 3 day culture with spleen cells.

Active material was consistently found in the L10 fraction (approximate mol. wt, on membrane filtration, 1000–10,000) from a spontaneous mouse tumour (SCS-3), a chemically-induced mouse tumour (A-2) and cell lines derived from human malignant melanoma (MM200) and breast cancer (T470), but not from mouse or guinea-pig fibroblasts. The L10 fraction had no direct inhibitory effect on migration, whereas the L1 fraction did. The migration inhibitor described by Pike & Snyderman (1976), however, had an apparent mol. wt of 6000 to 10,000. The discrepancy may be due to the use of culture supernatants rather than extracts and perhaps to differences in fractionation procedures. Moreover, significant direct suppression of chemotactic migration was only obtained here when the L1 inhibitor was incubated with LDCF in the lower well of the chemotaxis chamber. Incubation with macrophages in the upper well produced little or no inhibition. The mechanism of direct inhibition is not clear. However, as inhibition occurred when the L1 fraction was mixed with the chemotactic stimuli, it could act by blocking binding of the chemoattractant to the surface receptor on the macrophage.

Previous studies in this laboratory had shown that tumour cell supernatants had no selective effect on mitogen induced proliferation of mouse spleen cells, some inhibition being produced by both tumour cells and normal cells (Nelson & Nelson, 1978). The present experiments, however, showed a selective inhibition of LDCF production, perhaps reflecting the dissociation between proliferation and lymphokine production (Gately & Mayer, 1978).

Inhibition of lymphokine production did not appear to involve binding of the active material to PHA. It was most marked when the inhibitor was present from the start of the spleen cell culture. This suggested that it might act mainly on the early events of lymphocyte stimulation. It was then found that the active material also inhibited the production of IL1 by thioglycollate-induced macrophages, though not by *C. parvum* activated macrophages. Although the connection between the two phenomena is not clear, activated macrophages have been found to resist the phagocytosis-inhibitory effects of tumour supernatants and their L1 fractions (M. Nelson & D. S. Nelson, submitted for publication).

The production of LDCF requires macrophages (Wahl, Wilton, Rosenstreich & Oppenheim, 1975) which act in part by producing IL1 (Oppenheim, Mizel & Meltzer, 1979). Therefore, by implication, inhibition of IL1 production by the L10 tumour fraction indicates that it acts on one of the two signals provided by macrophages in T-cell activation (Mizel & Ben-Zvi, 1980) in the production of LDCF. The identification of the macrophage as the target cell does not, however, exclude the possibility that T cells can also be the target cells of the L10 inhibitor. Time course experiments (Fig. 1) showed that the L10 inhibitor acted at the early stages of lymphocyte activation. These findings are in agreement with those of Wahl *et al.* (1975) indicating that macrophages were involved in the earliest events of lymphocyte activation in the production of guinea-pig LDCF.

Matsubara, Suzuki, Nakamura, Edo & Ishida (1980) reported that the Ehrlich ascites tumour impaired Type II interferon production but we are unaware of other studies of the effects of tumour cells or products on lymphokine formation and have not systematically studied other lymphokines.

The possible role of lactic dehydrogenase (LDH) virus in the tumours used has been discussed in previous reports (Nelson & Nelson, 1980; Farram, Nelson & Nelson, 1981). Its presence in the tumours used was not detected, although the role of other viruses cannot be excluded. However, the presence of inhibitors in supernatants from the human tumour cell lines MM200 and T470 argues against a role of murine viruses in determining macrophage or lymphocyte function in these studies.

There are several parallels between the inhibition of the production of the lymphokine LDCF and the inhibition of DTH *in vivo*. Both are tumour selective, both lack species specificity and both are age-dependent (Nelson & Nelson, 1978, 1980). Both are produced by material which binds to Con A-Sepharose. The hypothesis that the inhibition of DTH is partly caused by inhibition of LDCF production is therefore tenable. Furthermore, inhibition of LDCF production may in turn be caused partly or wholly by inhibition of the production of another cytokine, the monokine IL1. These findings can, however, offer only a partial explanation for the effects of tumour supernatants on DTH. The L10 fraction inhibits only the early phase of DTH reactions. The G10 fraction, however, inhibits only the later phase and its mode of action remains unknown. It is also not known whether similar explanations will apply to immunologically non-specific mononuclear cellular inflammatory reactions.

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