Eur J Cancer Clin Oncol, Vol. 18, No. 3, pp. 307–315, 1982
Printed in Great Britain.

0277-5379/82/030307-09$03.00/0

Rous Sarcoma Virus-induced Tumors in Mice—I. Macrophage-mediated Natural Cytotoxicity*

MIRELLA GIOVARELLI,† SANTO LANDOLFO,† ALAN C. WHITMORE‡ and GUIDO FORNI†
†*Dept. of Microbiology, University of Torino, Via Santana* 9, 10126 *Torino, Italy and* ‡*Dept. of Immunology, Duke University, Chapel Hill, NC* 27710, *U.S.A.*

Abstract—*Lymphoid cells from normal untreated A.SW mice showed a cell-mediated cytotoxicity (CMC) towards Rous sarcoma virus-induced tumor lines in a* 48-*hr microcytotoxicity assay* in vitro, *using* ^{3}[H]-*thymidine prelabeled tumor cells. The tissue distribution of this natural CMC shows high levels of activity in the peritoneal cavity, intermediate levels in bone marrow and spleen, and low levels in lymph node, peripheral blood and thymus. Natural CMC of unstimulated resident peritoneal cells against tumor cells was also found in several strains of mice. This was not dependent on the donor's age, since similar levels appeared in mice aged* 1–40 *weeks. Passage through nylon wool columns or adherence to the plastic surface of resident peritoneal cells removed cytotoxic activity. By contrast, adherent cells displayed significant CMC levels. Treatment with carbonyl iron powder and magnet decreased peritoneal cell cytotoxicity when performed once and abolished it when repeated. All these results suggest that in mice, natural cytotoxicity against Rous sarcoma virus-induced tumors is primarily mediated by macrophages or macrophage-like cells.*

INTRODUCTION

NATURAL cell-mediated immunity embraces a wide group of cytotoxic mechanisms that are virtually active before the arrival of target cells, or are quickly activated by them. These cytotoxic mechanisms often appear to have an important role in immunosurveillance and resistance to foreign cells, tumors or microorganisms (for a review see [1]). They differ from T cell-mediated cytotoxicity in that they are fully present in unimmunized individuals, are not thymus dependent and are less restricted with regard to their specificity [2]. The cells responsible are heterogeneous not only among various species but also with respect to various targets [3]. In the mouse, a particular class of lymphocytes (NK and NC cells) exhibit spontaneous cytotoxicity to several lymphoma or solid tumors in short-term assays [2, 4]. Spontaneous cytotoxicity can also be mediated by a subpopulation of adhering non-phagocytic peritoneal B lymphocytes [5]. Lastly, cells belonging to the monocyte–macrophage lineage, (henceforth referred as Mø), harvested without deliberate stimulation, express a powerful cytolytic and cytostatic activity against a variety of tumor and parasitic cells [6–8].

The present studies were undertaken to test the occurrence and characteristics of the cells involved in natural cell-mediated cytotoxicity (CMC) towards tumors induced by Rous sarcoma virus (RSV) in mice. This tumoral system is of particular interest since the RSV genome consists of only a few genes, of which the *src* gene is solely involved in the induction and maintenance of neoplastic transformation. The molecular biology of cell membrane antigens appearing after RSV transformation and their relationship with neoplastic cell behavior are under intensive investigation [9].

In the mouse, susceptibility to RSV tumorigenesis is influenced by a dominant gene, or genes, associated with the major histocompatibility complex (H-2) [10], and is inversely correlated with the ability to mount a tumor-neutralizing immune response [11]. Incidence and latency of primary RSV-induced tumors are also influenced by immunological manipulation.

This paper describes the tissue, strain, age distribution and some characteristics of the cells responsible for CMC towards RSV-induced tumor lines in syngeneic mice.

Accepted 13 November 1981.
*This work was supported by grants from the Italian National Research Council (CNR PFCCN No. 80.01548.96) and the Assoc. Ital. Cancro.

MATERIALS AND METHODS

Mice

BALB/c, C57BL/6 and C3H mice were purchased from Charles River Laboratories (Calco, Italy). B10.PL, B10.RIII and B10.BR mice were a generous gift from Dr. G. Parmiani, Ist. Naz. Tumori, Milano, Italy; C3H/HeJ mice were a generous gift from Dr. L. Ruco, Ist. Anat. Patologica, Rome, Italy. All other strains of mice were bred in our animal facilities from breeders obtained as follows: B10.S(12R), B10.HTT and A.TFR1 from Dr. C. David, Mayo Clinic, Rochester, MN; A.WySn and A.BY from Dr. G. Haughton, University of North Carolina, Chapel Hill, NC; A.TH, A.TL and A.SW from Dr. D. C. Shreffler, Washington University, St. Louis, MO; CBA/J and CBA/N from Dr. I. Scher, Naval Medical Research Institute, Bethesda. MD. Male and female 6–7-week-old mice were employed, except where otherwise specified.

Tumors

Two sarcomas (RS-33 and RS-46) were induced by injecting the Schmidt–Ruppin strain of RSV in newborn (less than 3 days old) A.SW mice, as previously described in detail [10]. From the third *in vivo* passage of the tumor were established *in vitro* lines by treatment with trypsin and DNase, as previously described in detail [12]. Early *in vitro* passages were cloned and the cells were subsequently maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 2 mM glutamine, 50 μg/ml gentamycin, 100 μg/ml streptomycin, 100 U/ml penicillin and 20 U/ml mycostatin. When injected subcutaneously in syngeneic A.SW mice, the cells give rise to tumors resembling the original tumor in growth pattern and histological characteristics. Normal BALB/c 3T3 fibroblasts (B/C-3T3), and B/C-3T3 transformed by the B77 substrain of RSV by Dr. Bishop *et al.* [13], were kindly provided by Dr. M. Prat, Dept. of Histology, Univ. of Trieste, Italy. The Moloney virus-induced leukemia of A.SW mice (YWA) was kindly provided by Dr. G. Klein, Karolinska Institute, Stockholm, Sweden. The Moloney virus-induced lymphoma of A/WySn mice (YAC-1) was kindly provided by Dr. A. Santoni, Univ. Perugia, Italy. All these cells were also cultured in RPMI 1640 supplemented as described above.

Labeling of target cells. The *in vitro* lines were labeled with Na chromate [^{51}Cr] or [^{3}H]-thymidine ([^{3}H]-TdR). For [^{51}Cr] labeling, the monolayers were incubated with 5 ml of trypsin–EDTA for 5 min at 37°C. After two washings, 5×10^6 cells were incubated with 200 μCi of [^{51}Cr] (specific activity 300 mCi/mg, Sorin, Saluggia, Italy) for 40 min at 37°C, then washed and adjusted to a concentration of 1×10^5/ml. Cells were labeled with [^{3}H]-TdR by incubating non-confluent monolayers in 75 cm^2 tissue culture flasks (Falcon, Oxnard, CA) for 24 hr with 20 ml of medium containing 0.5 μCi/ml of [^{3}H]-TdR (26 Ci/mM, Sorin). Target cells were then harvested by exposure to 5 ml of trypsin–EDTA for 5 min at 37°C, washed twice and resuspended to a concentration of 1×10^5 cells/ml in the test medium.

Effector cells. Spleens, lymph nodes (mesenteric and cervical) and thymuses were removed aseptically and teased apart into cold buffered salt solution (BSS) supplemented with 5% FBS. Bone marrow cells were obtained from long bones by flushing the cavity with cold BSS. Mononuclear peripheral blood leukocytes were isolated by centrifugation over a cushion of Ficoll–Hypaque (Nyegaard and Co., Oslo, Norway), as described by Böyum [14]. Peritoneal cells (PC) were obtained from normal untreated mice. In all the strains studied, the mean recovery ranged between $2–3 \times 10^6$ cells/mouse. Differential cell counts made on Diff Quick (Hartleco, Gibbstown, NY)—stained cell smears showed that this cell population consists of approximately 55–65% Mø, 5–10% neutrophils and 15–20% lymphocytes. Moreover, 70–75% of the cells ingested latex beads (Dow Chemical Co., Midland, MI) after overnight incubation at 37°C with constant rotation. No variations exceeding ±5% of these values were observed in any strains.

The cell suspensions were filtered through sterile gauze and, unless otherwise specified, treated with Tris-buffered ammonium chloride to lyse red blood cells, filtered again, washed twice and resuspended in test medium at suitable concentrations.

Adherence to plastic surfaces. Five milliliters of peritoneal cell suspensions (5×10^6 cells/ml) were plated on 100 mm plastic Petri dishes (No. 3003; Falcon, Oxnard, CA). After incubation in RPMI 1640 plus 10% FBS for 3 hr at 37°C, the nonadherent cells were collected by four vigorous washes with BSS and adherent cells were dislodged with a rubber policeman in the presence of chilled BSS with 10% FBS. The viability of the adherent cell population obtained was evaluated as about 50–60% by the trypan blue exclusion test. This population was enriched to about 80% of latex-ingesting cells. By contrast, the nonadherent population contained less than 5% latex-ingesting cells.

Nylon wool columns. Peritoneal cell populations were fractionated on nylon wool columns, as described by Julius *et al.* [15]. The column-emerging cells consist of 90% Thy 1.2-positive cells, as determined by cytotoxicity with anti-Thy 1.2 serum and rabbit complement (generously donated by Dr. H. T. Holden, NCI, Bethesda, MD). Adherent cells were recovered by transferring the nylon wool to a Petri dish containing chilled BSS and teasing them apart with sterile forceps.

Carbonyl iron powder and magnet. Removal of phagocytic cells from peritoneal cell population by carbonyl iron powder and magnet was performed as previously described [16]. After one treatment, latex-ingesting cells decreased to about 15–20%. In some experiments, the peritoneal cells were treated twice consecutively with carbonyl iron powder and magnet. In this case, latex-ingesting cells decreased to less than 5%.

Cytotoxicity assays. Cytotoxic activity of various lymphoid cell suspensions was tested in 4- and 18-hr assays by using [^{51}Cr]-labeled target cells, and in 48-hr assays by using [^{3}H]-TdR-labeled cells. The [^{51}Cr] release assay was performed in round-bottomed microtiter plates (Sterilin, Teddington, Middx., U.K.) by adding progressive numbers of effector cells to various target cells (10^4/well) in a final volume of 0.2 ml of RPMI 1640 medium, supplemented as above. The plates were spun down at 200 *g* for 1 min, incubated in 5% CO_2 atmosphere at 37°C for 4 or 18 hr and then spun down at 400 *g* for 10 min. The radioactivity of 0.1 ml of supernatant was determined in a gamma counter (Packard, Downer's Grove, IL). Spontaneous release was determined by incubating target cells in the medium only. Maximum release was determined by exposure of targets to 0.25% Triton X-100. [^{3}H]-TdR release was tested in flat-bottomed microtiter plates (No. 3040, Falcon, Oxnard, CA) by using RPMI 1640 medium enriched as above and additionally supplemented with 5×10^{-5} M 2-mercaptoethanol unless otherwise specified. As previously described in detail [8], 0.1 ml of a suspension of labeled target cells (2×10^5/ml) was placed in each well, followed by 0.1 ml of a suspension containing progressive numbers of effector cells. Spontaneous release was determined by adding 0.1 ml of medium alone. Maximum release was determined by adding 0.1 ml of 10% sodium dodecyl sulfate (SDS) in water. It was about 85 ± 5% of the total radioactivity incorporated, as evaluated by solubilizing the same number of labeled tumor cells with NCS (Hopkin and Williams, Chadwell Heath, U.K.) directly in the scintillation vials. The plates were incubated at 37°C in 5% CO_2 atmosphere and after 48 hr were centrifuged at 400 *g* for 10 min. The supernatant (0.1 ml) was transferred to scintillation vials. A volume of 2 ml of Picofluor 15 (Packard) was added and the radioactivity determined in a Packard Mark II liquid scintillation spectrometer. Percentage of cytotoxicity in both [^{51}Cr] and [^{3}H]-TdR release assays was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{cpm of experimental release} - \text{cpm of spontaneous release}}{\text{cpm of maximum release} - \text{cpm of spontaneous release}} \times 100.$$

The values of maximum release ranged from 9300 to 25,000 cpm. Spontaneous release lay within 12–17% of the maximum release. Experiments resulting in values outside these limits were discarded. The results are expressed as the arithmetic mean of triplicate cultures per experimental group ± S.D. The intraexperimental variation never exceeded 8%. Except when otherwise specified (Table 4), each experiment was performed at least 5 times. One or two representative results are reported in the tables.

RESULTS

Short-term cytotoxicity

Initially, we performed experiments to determine whether normal spleen cells from low NK-reactive A.SW and high NK-reactive CBA/J mice [17] exhibit natural CMC in 4 or 18 hr cytotoxicity assays. By 4 hr, the release of [^{51}Cr] from prelabeled RSV-induced tumor cells in the presence of both kinds of spleen

Table 1. Failure to detect NK-like activity to RSV-induced tumors in 4-hr cytotoxicity test

Spleen cells from:	Tumor target*	Percentage of cytolysis (E : T ratios) 150 : 1	75 : 1	37.5 : 1
A.SW	RS.33	0.8	−3.4	−2.1
CBA/J	RS.33	2.1	−1.4	0.8
A.SW	RS.46	−1.6	0.3	0.5
CBA/J	RS.46	1.0	−1.1	0.2
A.SW	YAC-1	5.0	3.1	2.4
CBA/J	YAC-1	43.3	34.0	22.0

*Cells labeled with [^{51}Cr].

Table 2. Natural cell-mediated cytotoxicity of cells from various tissues of normal A.SW mice against RS-33 tumor cells

Source of effector cells	No. of experiments	Percentage of cytotoxicity* 50 : 1†	25 : 1	12 : 1
Thymus	7	0.5 ± 0.2	0.1 ± 0.2	0.1 ± 0.2
Peripheral blood	4	3.3 ± 1.2	2.6 ± 1.2	2.4 ± 1.2
Lymph nodes	7	4.8 ± 2.0	4.2 ± 1.2	2.4 ± 0.2
Spleen	9	17.0 ± 6.4	14.2 ± 4.8	11.6 ± 5.0
Bone marrow	9	18.6 ± 4.4	16.4 ± 6.0	15.1 ± 4.1
Peritoneum	10	21.4 ± 7.0	28.8 ± 2.8	22.5 ± 3.1

*Mean percentage cytotoxicity of various experiments (each performed in triplicate) ±S.E. against tumor cells labeled with [^{3}H]-TdR.
†E : T ratios.

cells was not significantly higher than from the same targets incubated in medium only (Table 1). Similar results were obtained after 18 hr (data not shown). By contrast, incubation of YAC-1 lymphoma target cells with CBA/J spleen cells resulted in a marked cytolysis at all effector : target (E : T) ratios tested. Lower cytotoxicity was found in the presence of A.SW spleen cells.

Tissue distribution of long-term cytotoxicity

Since no NK-line cytotoxicity to RSV-induced tumor cells was found in short-term tests, we evaluated the presence of natural CMC in 48 hr assays. Table 2 shows that lymphoid cells from normal A.SW mice are cytotoxic against [^{3}H]-TdR-labeled RS-33 tumor cells at E : T ratios of 50 : 1, 25 : 1, 12 : 1. The tissue distribution of this natural cytotoxicity shows that peritoneal cells, harvested without any deliberate stimulation or activation, were the most cytotoxic, followed by bone marrow and spleen cells. Lymph node and peripheral blood leukocytes have very little, if any, detectable cytotoxicity. This rank order was the same at all three E : T ratios tested and was consistently observed every time the experiment was repeated.

Kinetics and influence of environmental conditions

Since peritoneal cells display the highest levels of natural cytotoxicity, we decided to study their activity in detail. Its kinetics showed that cytolysis was low after the first 24 hr, increased by 48 hr and remained at the same levels after 72 hr (Fig. 1). This pattern of lysis runs counter to the view that tumor cell destruction depends on drastic depletion of nutritive constituents. Moreover, similar levels of cytotoxicity at 48 and 72 hr were observed when 0.1 ml of medium was carefully removed from each well and replaced with fresh medium after 24 and 48 hr of culture (data not shown). To rule out the possibility that the presence of endotoxin in FBS or FBS by itself [18, 19] could play a significant role in triggering the observed cytotoxicity, we performed parallel tests with a medium containing LPS-free FBS, as assessed by the Lymulus amebocyte lysate assay (kindly donated by Dr. A. Mantovani, Ist. M. Negri, Milan, Italy), with a medium containing the batch of FBS used in the previous tests and a medium containing 1% fresh A.SW serum only. In addition, before use in this assay, the target cells were washed several times to remove most of the FBS components. Comparable levels of cytolysis can be seen in the two representative experiments in Table 3.

Strain distribution

It has been reported that natural resistance to tumors varies widely under the influence of genes mapping both inside and outside the

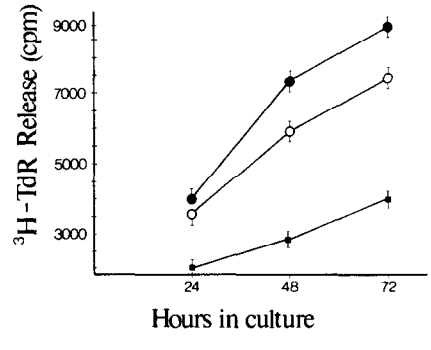

Fig. 1. Kinetics of the cytolytic activity of peritoneal cells against RS-33 target fibroblasts. A representative experiment is reported. Mean cpm *of* [^{3}H]-*TdR release by triplicate cultures at E : T ratios of* 12 : 1 (●—●) *and* 6 : 1 (○—○). (■—■) *shows the release of the label in the absence of effector cells. Vertical bars:* ±*S.D. The maximum release was* 21,446 cpm.

Table 3. Natural cell-mediated cytotoxicity of peritoneal cells against RS-33 tumor cells in medium supplemented with different sera

Source of serum	E : T Ratios	Percentage of cytotoxicity* Experiment 1	Experiment 2
10% FBS	25 : 1	20.2 ± 1.3	25.4 ± 1.5
	12 : 1	18.8 ± 1.2	23.8 ± 1.6
10% LPS free FBS	25 : 1	26.0 ± 2.0	30.0 ± 2.1
	12 : 1	21.4 ± 1.4	24.6 ± 1.6
1% Fresh mouse serum	25 : 1	26.6 ± 1.7	26.0 ± 1.4
	12 : 1	20.5 ± 1.2	24.0 ± 1.5

*Mean percent cytotoxicity (three replicates per experiment) ±S.D.

Table 4. Natural cell-mediated cytotoxicity of peritoneal cells from various mouse strains against RS-33 tumor cells

Strain	H-2 haplotype	Background genes	No. of experiments	Percentage of cytotoxicity* 25 : 1†	12 : 1	6 : 1
A.SW	s	A	10	21.4 ± 7.0	28.8 ± 2.8	22.5 ± 3.1
A.TL	tl	A	4	13.3 ± 6.4	20.8 ± 6.2	18.8 ± 7.2
A.TH	t2	A	4	21.8 ± 7.1	18.6 ± 10.0	8.8 ± 6.0
A.WySn	a	A	3	19.4 ± 8.0	26.2 ± 7.0	9.6 ± 2.7
A.By	b	A	3	21.3 ± 6.2	30.2 ± 5.0	16.2 ± 4.3
A.TFR1	an	A	3	20.1 ± 8.0	27.2 ± 5.6	13.3 ± 4.1
B10.BR	k	B10	4	26.6 ± 6.5	32.8 ± 9.0	25.6 ± 8.5
B10.BR	s	B10	3	15.6 ± 5.0	27.8 ± 6.2	13.9 ± 4.1
B10.RIII	r	B10	3	18.4 ± 5.0	25.6 ± 7.4	10.0 ± 6.1
B10.PL	u	B10	3	27.0 ± 7.9	20.0 ± 2.9	12.6 ± 3.8
B10.HTT	t3	B10	3	22.6 ± 9.9	26.7 ± 7.8	21.4 ± 8.0
C57BL/6	b	B6	3	16.2 ± 3.5	20.5 ± 2.0	10.1 ± 3.2
C3H/CR	k	C3H	3	25.2 ± 8.8	28.0 ± 7.0	20.0 ± 6.1
C3H/HeJ	k	C3H	2	22.5 ± 6.4	25.2 ± 6.2	14.2 ± 5.1
CBA/N	k	CBA	2	26.1 ± 7.1	25.3 ± 3.8	19.2 ± 4.5
CBA/J	k	CBA	2	27.5 ± 5.0	20.1 ± 4.5	14.0 ± 3.6
BALB/c	d	BALB	16	18.4 ± 6.2	27.9 ± 3.0	20.0 ± 4.5

*†As for Table 2.

H-2 complex [19, 20]. It was therefore of interest to compare the levels of CMC to RSV-induced tumors of A.SW mice with that of mice differing at H-2 and background genes. As shown in Table 4, quite surprisingly, no significant variations were found. In addition, no differences were observed between C3H/CR and C3H/HeJ mice, even though C3H/HeJ bear a gene-coded defect in LPS- and lymphokine-dependent activation of macrophage cytotoxicity [21]. Comparable natural cytotoxicity levels were also observed in CBA/J and CBA/N mice. CBA/N mice are a mutant line of CBA strain bearing an X-linked immune defect (*xid*) that results in the arrest of B lymphocyte function and maturation [22]. A similar pattern of cytotoxicity was observed against RS-46 tumor cells (data not shown).

Effect of donor age

Donor age has great influence on the cytotoxicity levels in some CMC systems [23, 24], though not in all [4, 25]. In our system, in fact, comparable levels of cytotoxicity were displayed by peritoneal cells from 1- to 40-week-old A.SW donors (Table 5).

Role of adherent and phagocytic cells

In order to characterize the effector cells responsible for the natural CMC to RSV tumor cells, we tested the effect of fractionating adherent and nonadherent peritoneal leukocytes. Table 6 shows that passage through nylon wool columns or adherence to plastic surfaces almost completely removed cytotoxic activity. In both cases, it could be recovered in the adherent fraction. The non-complete recovery may, in

fact, be due solely to the damage caused by the mechanical manipulations used in cell separation [8]. A single treatment of peritoneal cells with carbonyl iron and magnet removes phagocytic cells and significantly reduces natural cytotoxicity, while it is completely abrogated by a double treatment. It should be pointed out that lymphocytes recovered after these two treatments still had high viability, as shown by the trypan blue exclusion test (90% dye-excluding cells), and were able to give a high blastic response to both phytohemagglutinin and lipopolysaccaride from *Escherichia coli* (data not shown).

The effector cells involved are thus both adherent and phagocytic, which suggests that they belong to the monocyte–macrophage lineage.

Table 5. Effect of donor age on natural cell-mediated cytotoxicity of normal A.SW peritoneal cells against RS-33 tumour cells

Donor age (weeks)	No. of mice*	E : T Ratios	Percentage of cytotoxicity† Experiment 1	Experiment 2
1	10	25 : 1	17.8 ± 0.5	16.4 ± 0.3
		12 : 1	25.2 ± 0.8	21.0 ± 0.2
4	12	25 : 1	12.7 ± 0.4	19.0 ± 0.5
		12 : 1	28.1 ± 1.2	23.5 ± 0.9
6–7	15	25 : 1	12.6 ± 0.6	14.3 ± 0.4
		12 : 1	26.4 ± 1.3	22.2 ± 0.3
18	12	25 : 1	14.2 ± 0.7	18.5 ± 0.2
		12 : 1	27.6 ± 0.9	23.1 ± 0.4
40–42	8	25 : 1	26.9 ± 1.0	20.2 ± 0.8
		12 : 1	23.2 ± 0.9	24.0 ± 0.3

*Number of mice used as source of peritoneal cells in each experiment.
†As for Table 3.

Selectivity of peritoneal cytotoxicity

The selectivity of peritoneal cell CMC was determined from their ability to lyse various transformed and normal cells. The two representative experiments reported in Table 7 demonstrate that comparable levels of cytolysis were observed against two A.SW and one BALB/c RSV-transformed fibroblasts, as well as towards A.SW leukemic cells transformed by Moloney sarcoma virus. No significant cytotoxicity was observed against normal BALB/c fibroblasts.

DISCUSSION

This study has shown that a marked natural CMC takes place *in vitro* against RSV-induced sarcomas. RSV-transformed fibroblasts are apparently unaffected by NK-like short-term cytotoxicity, but are killed in significant quan-

Table 6. Natural cytotoxicity of peritoneal cells from normal A.SW mice after separation of adherent and phagocytic cells

Cell fractionation	Percentage of cytotoxicity* 25 : 1		12 : 1	
None	20.6	(100.0)†	19.0	(100.0)
Cells eluting from nylon wool columns	3.0	(14.5)	1.5	(7.9)
Cells nonadhering to plastic surfaces	4.1	(19.9)	4.7	(24.7)
Cells adhering to plastic surfaces	23.0	(111.6)	24.7	(130.0)
Cells remaining after a single treatment with carbonyl iron and magnet	9.5	(46.1)	7.9	(41.6)
Cells remaining after two consecutive treatments with carbonyl iron and magnet	0.2	(0.9)	0.2	(1.0)

*As for Table 2.
†Values in parentheses are the percentage of residual cytotoxicity compared with untreated peritoneal cells at the corresponding E : T ratios.

Table 7. Cytotoxicity of peritoneal cells from normal A.SW mice against virus-transformed and normal target cells

Experiment	E : T Ratios	Percentage of cytotoxicity against:* RS-33†	RS-46†	3T3-B77†	B/C-3T3	YWA‡
	25 : 1	27.3	28.6	26.0	−2.0	29.1
1	12 : 1	31.0	30.4	20.3	0.1	32.2
	6 : 1	25.0	22.6	18.3	−6.4	21.1
	25 : 1	21.7	17.5	27.3	1.2	35.2
2	12 : 1	24.5	25.2	30.2	2.0	42.1
	6 : 1	17.4	20.5	16.4	1.1	26.3

*Mean percentage cytotoxicity of triplicate determinations. The standard deviation in these experiments never exceeded 5% and therefore is not reported.
†RSV-induced fibrosarcomas.
‡Moloney-induced leukemic cells.

tities after 48-hr culture in the presence of leukocytes from unstimulated normal mice.

The tissue distribution of this reactivity shows that unstimulated peritoneal cells are the most cytotoxic. Bone marrow and spleen cells display an intermediate activity, whereas that of peripheral blood and lymph node cells is much lower. No cytotoxic activity is displayed by thymus cells. This pattern is quite similar to that of the Mø-dependent cytotoxicity observed in several mouse and rat systems [6–8]. The adherent characteristics of the effector cells in the peritoneal cell population and the virtual abolition of cytotoxicity by magnetic removal of phagocytic cells after a double treatment with carbonyl iron and magnet clearly indicate that the natural CMC observed in the peritoneal cell population is attributable to cells belonging to monocyte–macrophage lineage. This identification is further supported by the kinetics of the cytocidal activity, which resemble that of Mø [6, 19, 25].

The natural CMC against RSV-induced tumors is neither significantly affected by donor age, nor under an appreciable genetic control of H-2 and background genes. In these two parameters, too, it is quite distinct from NK-dependent cytotoxicity [4, 17, 23, 24]. In effect, typical NK low-responder strains, such as A.WySn, A.BY and A.SW, display the same levels of cytotoxicity as high-responder strains, such as CBA/J, CBA/N, C3H, and C57Bl/6 [14, 19]. Also, strains which are highly susceptible to primary RSV tumor induction after neonatal injection (CBA, A.WySn, A.BY) have no less natural CMC activity than do highly resistant strains (A.SW, B10.S, BALB/c) [26].

Contrary to what has been reported by other workers [4, 25], the natural cytotoxicity observed in our system did not depend upon the environmental conditions in which donor mice were kept, since comparable levels were noted in mice from several laboratories (Ist. Naz. Tumori, Milan, Italy; Ist. Anat. Patol., Rome, Italy; Charles River Laboratories, Calco, Italy, as described in Materials and Methods) and killed immediately after arrival, and in mice bred or maintained in our animal facilities. The finding that peritoneal cell cytotoxicity is comparable in C3H/HeJ and C3H/CR mice, as well as in other strains, seems to preclude the possibility that endotoxin contamination of FBS was responsible, since C3H/HeJ cells are not activated by bacterial endotoxins [21]. In addition, natural CMC is not affected by the use of FBS endotoxin free as determined by the Lymulus assay, nor is it dependent on the presence of FBS in the culture medium, since it was also detected when 1% of fresh mouse serum only was added to the culture medium. Thus, natural CMC is not markedly influenced by experimental conditions. It reflects, rather, an Mø activity potentially present *in vivo* in conventionally kept mice in absence of overt experimental stimulation. However, we can not exclude that the targets themselves could play a positive role in triggering the Mø-mediated cytotoxicity. If this was the case, we would assume that only a restricted number of targets could do it.

There are conflicting reports in the literature concerning the ability of Mø to kill selectively transformed cells [7, 18, 27]. In our experiments, RSV-transformed fibroblasts and Moloney leukemia virus-induced lymphoma cells, but not contact-inhibited BC-3T3 fibroblasts, trigger or are susceptible to this CMC. Some specialized membrane structures [28] or

particularly intense mitotic behaviour and metabolic activity of the targets (L. Varesio, personal communication) could act as triggering signals. In this context, it is of particular interest to consider the parallelism between our present findings and those we have recently obtained in a non-tumoral system [8] by using the same experimental conditions. We observed that the natural CMC displayed by normal mice against an extracellular protozoon, *Trichomonas vaginalis*, is very similar in tissue distribution, age independence and characteristics of its effector cells, though not in genetic control, to the one displayed against tumor cells.

In conclusion, our present findings on RSV-induced sarcomas, in accord to what has been observed in several distinct tumor systems [27, 29], suggest that natural activity of Mø is not confined to bacteria and detritus phagocytosis but appears to be a true surveillance mechanism endowed with broad specificity. The particular social position of Mø within the immune system allows their weight *in vitro* to be modulated by various lymphocyte signals sent quickly after specific and genetic restricted antigen recognition [30].

Acknowledgement—We wish to thank Dr. John Iliffe for careful review of the manuscript.

REFERENCES

1. CUDKOWICZ GM, LANDY M, SHEARER GM. *Natural Resistance Systems Against Foreign Cells, Tumors and Microbes.* New York, Academic Press, 1978.
2. HERBERMAN RB, HOLDEN HT. Natural cell-mediated immunity. *Adv Cancer Res* 1978, **27**, 305–378.
3. WELSH RM. Mouse natural killer cells: induction, specificity, and function. *J Immunol* 1978, **121**, 1631–1635.
4. STUTMAN O, PAIGE JC, FIGARELLA EF. Natural cytotoxic cells against solid tumors in mice. I. Strain and age distribution and target cell susceptibility. *J Immunol* 1978, **121**, 1819–1827.
5. NATHAN GF, HILL VM, TERRY WD. Isolation of a subpopulation of adherent peritoneal cells with anti-tumor activity. *Nature (Lond)* 1976, **260**, 146–148.
6. KELLER R. Macrophage-mediated natural cytotoxicity against various target cells *in vitro*. I. Macrophages from diverse anatomical sites and different strains of rats and mice. *Br J Cancer* 1978, **37**, 732–741.
7. TAGLIABUE A, MANTOVANI A, KILGALLEN M, HERBERMAN RB, MCCOY JL. Natural cytotoxicity of mouse monocytes and macrophages. *J Immunol* 1979, **122**, 2363–2370.
8. LANDOLFO S, MARTINOTTI MG, MARTINETTO P, FORNI G. Natural cell-mediated cytotoxicity against *Trichomonas vaginalis* in the mouse. I. Tissue, strain, age distribution and some characteristics of the effector cells. *J Immunol* 1980, **124**, 508–514.
9. COMOGLIO PM, PRAT M, BERTINI M. Tumor specific cell surface antigens of Rous sarcoma virus-transformed mammalian fibroblasts. In: ARNON R, SPREAFICO F, eds. *Tumor-associated Antigens and Their Specific Immune Response.* London, Academic Press, 1979, 1–20.
10. WHITMORE AC, HAUGHTON G. Genetic control of susceptibility of mice to Rous Sarcoma virus tumorigenesis. I. Tumor incidence in inbred strains and F_1 hybrids. *Immunogenetics* 1975, **2**, 379–388.
11. BANKS RA, BABCOCK GF, WHITMORE AC, HAUGHTON G. Effect of immunologic intervention on *in vivo* murine Rous sarcoma virus tumorigenesis. *J Natl Cancer Inst* 1979, **63**, 1423–1431.
12. FORNI G, VARESIO L, GIOVARELLI M, CAVALLO G. Dynamic state of spontaneous immune reactivity towards a mammary adenocarcinoma. In: ARNON, R, SPREAFICO F, eds. *Tumor-associated Antigens and Their Specific Immune Response.* London, Academic Press, 1979, 167–192.
13. BISHOP M, WEISS SR, OFFERMANN OH *et al.* The strategy of retrovirus gene expression. In: BARLATI S, DE GIULI MORGHEN C, eds. *Avian RNA Tumor Viruses.* Piccin Medical Book, 1978, 81–189.
14. BÖYUM A. Separation of leukocytes from blood and bone marrow. *Scand J Clin Lab Invest.* 1968, **21**, 97–102.
15. JULIUS ME, SIMPSON E, HERZENBERG LW. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur J Immunol* 1973, **3**, 645–669.

16. LANDOLFO S, HERBERMAN RB, HOLDEN HT. Macrophage–lymphocyte interaction in migration inhibition factor (MIF) production against soluble or cellular tumor-associated antigens. I. Characteristics and genetic control of two different mechanisms of stimulating MIF production. *J Immunol* 1978, **121**, 695–702.
17. RICCARDI C, SANTONI A, BARLOZZARI T, PUCCETTI P, HERBERMAN RB. *In vivo* natural reactivity of mice against tumor cells. *Int J Cancer* 1980, **25**, 475–486.
18. HIBBS JB, TAINTOR RR, CHAPMAN HA, WEINBERG JB. Macrophage tumor killing: influence of the local environment. *Science* 1977, **197**, 279–282.
19. PELS E, DEN OTTER W. Natural cytotoxic macrophages in the peritoneal cavity of mice. *Br J Cancer* 1979, **40**, 856–860.
20. STUTMAN O, CUTTITO MJ. Genetic influences affecting natural cytotoxicity (NC) cells in mice. In: HERBERMAN RB, ed. *Natural Cell Mediated Immunity Against Tumors.* New York, Academic Press, 1980, 431–442.
21. MELTZER M, RUCO L. Defective tumoricidal capacity of macrophages from C3H/HeJ mice. *J Immunol* 1978, **120**, 329–334.
22. SCHER I, AHMED A, STRONG DM, STEINBERG AD, PAUL WE. X-linked B lymphocyte immune defect in CBA/HN mice. I. Studies of the function and composition of spleen cells. *J Exp Med* 1975, **141**, 788–796.
23. HERBERMAN RB, NUNN ME, LAVRIN DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. *Int J Cancer* 1975, **16**, 216–218.
24. KIESSLING RE, KLEIN E, PROSS H, WIGZELL H. Natural killer in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cells. *Eur J Immunol* 1975, **5**, 117–121.
25. MELTZER MS. Tumoricidal responses *in vitro* of peritoneal macrophages from conventionally housed and germ free nude mice. *Cell Immunol* 1976, **22**, 176–181.
26. WHITMORE AC, BABCOCK GF, HAUGHTON G. Genetic control of susceptibility of mice to Rous sarcoma virus tumorigenesis. II. Segregation analysis of strain. A.SW-associated resistance to primary tumor induction. *J Immunol* 1978, **123**, 213–220.
27. ADAMS DO, SNYDERMAN S. Do macrophages destroy nascent tumors? *J Natl Cancer Inst* 1979, **62**, 1341–1345.
28. KELLER R. Regulatory capacities of mononuclear phagocytes with particular reference to natural immunity against tumors. In: HERBERMAN RB, ed. *Natural Cell-mediated Immunity Against Tumors.* New York, Academic Press, 1980, 1219–1270.
29. CHOW DA, GREENE MI, GRENE AH. Macrophage-dependent, NK independent "natural" surveillance of tumors in syngeneic mice. *Int J Cancer* 1979, **23**, 788–797.
30. VARESIO L, LANDOLFO S, GIOVARELLI M, FORNI G. The macrophage as the social interconnection within the immune system. *Dev Comp Immunol* 1980, **4**, 11–19.