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In vitro generated mast cells express natural cytotoxicity against tumour cells

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Summary. Bone marrow cells from mice were cultured *in vitro* in the presence of interleukin-3 (IL-3). After 2 weeks, the majority of the cells differentiated towards mast cells as judged by morphological and biochemical criteria. When populations of 99% IL-3-dependent mast cells (IL-3 MC) were tested for their anti-tumour activity *in vitro*, it was found that they can express natural cytotoxicity (NC) but not other natural reactivities. Moreover, the effector cells were not positive for Thy 1, Lyt 1 and Lyt 2 markers. The capability of mast cells to express NC activity seems to be related to their *in vitro* differentiation, since mast cell deficient W/W^v mice had normal NC activity. Thus, IL-3 MC must be added to the variety of cells capable of expressing natural anti-tumour reactivities *in vitro*.

INTRODUCTION

In vitro spontaneous cytotoxicity against tumour cells is expressed by a variety of effector cells, including lymphocytes (Herberman *et al.*, 1975; Kiessling *et al.*, 1975), macrophages (Tagliabue *et al.*, 1979) and mast cells (Farram & Nelson, 1980). Moreover, heterogeneity of the effectors of natural resistance has been shown to exist also within the lymphoid lineage (reviewed in Herberman, 1982). In fact, the phenotype of the killer lymphocyte varies according to the tumour target employed (Lust *et al.*, 1981; Minato, Reid & Bloom, 1981). Moreover, the phenotype of the effector lymphocytes can also differ when the same tumour target is employed if they originate from different anatomical sites (Tagliabue *et al.*, 1982). Thus, the heterogeneity of the cells involved in natural resistance has become a key issue in understanding the biology of this phenomenon, which is thought to play an important role in immunosurveillance.

One of the approaches employed to clarify this heterogeneity has been to test the cytotoxic activity of cells differentiated and immortalized *in vitro* with the aid of different growth factors. Interleukin-2 was the first and most widely used lymphokine for this purpose. However, more recently, much attention has also been focused on interleukin-3 (IL-3). In fact, this lymphokine, which was first described for its capability of inducing the 20α-hydroxysteroid dehydrogenase enzyme on lymphocytes (Ihle, Pepersack & Rebar, 1981), has been shown to possess multiple activities, including the property of selectively boosting natural cytotoxicity (NC) (Lattime, Pecoraro & Stutman, 1983) and the capability of promoting the *in vitro* growth of mast cells and basophils (Ihle *et al.* 1983). Here, we employed IL-3 to induce the differentiation of murine bone marrow cells to mast cells, and then we tested the capability of these cultured cells to express cytotoxic activities against tumour cells.

Abbreviations: C, complement; IL-3, interleukin-3; IL-3 MC, interleukin-3-dependent mast cells; LGL, large granular lymphocytes; LTC_4, leukotriene C_4; NC, natural cytotoxicity; NK, natural killer; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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MATERIALS AND METHODS

Mice

Inbred C3H/HeN CrlBR mice, obtained from the Charles River Breeding Laboratories (Calco, Italy), were bred in our animal facilities, as well as WBB6F$_1$-W/W^v mice and their littermates (WBB6F$_1$+/+, WBB6F$_1$-W/+ and WBB6F$_1$-W^v/+) derived from parental stocks WB/ReJ-W/+ and C57BL/6J-W^v/+ purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were employed at 8 weeks of age.

Culture of IL-3 dependent cells

Bone marrow cells obtained from C3H/HeN mice were cultured in 75 cm^2 flasks (Corning Glass Works, Corning, NY) kept upright at 37° in a humidified atmosphere with 5% CO_2. Each flask contained 30 ml of bone marrow cells at a starting density of 10^6 cells/ml in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sera-Lab, Crawley Down, Sussex, U.K.), 50 μg/ml gentamycin (Schering, Kenilworth, NJ), 25 mM HEPES buffer and 2 mM L-glutamine (hereafter referred to as complete medium). This medium was also supplemented with 50 μM 2-mercaptoethanol and 20% crude supernatant of WEHI-3 cells (obtained from Dr B. Bloom, Albert Einstein College of Medicine, Bronx, NY) cultured for 48 hr at 2×10^6 cells/ml in complete medium containing 1% FBS. The presence of IL-3 in WEHI-3 cell supernatant was assessed as the capability of inducing the enzyme 20α-hydroxysteroid dehydrogenase in cultures of nu/nu splenocytes (Ihle *et al.*, 1981; Tagliabue *et al.*, 1984).

Electron microscopy

In order to perform the analysis by transmission electron microscopy (TEM), 5×10^6 cells were fixed with glutaraldehyde, 2·5%, in phosphate buffer, 0·1 M, and post-fixed in 2% OsO_4 in the same buffer, dehydrated in ethanol and embedded in EPON 812. Ultrathin sections stained with uranyl acetate and lead citrate were observed under a Philips 301 or a Siemens Elmiskop Ia electron microscope. For scanning electron microscopy (SEM), the cells were fixed with glutaraldehyde, 2·5%, in phosphate buffer, 0·1 M. Dehydration with ethanol was followed by drying at the critical point with CO_2. After coating with gold in a high vacuum evaporator on a rotating-tilting stage, the samples were observed under a ETEC-Siemens U-1 scanning electron microscope.

Biochemical characterization of IL-3-dependent cells

Histamine content of cultured cells was assessed by fluorometric procedure as described by Anton & Sayre (1969). The release of leukotriene C_4 (LTC_4) was determined by radioimmunoassay with commercially available kits (New England Nuclear, Boston, MA) after IgE/anti-IgE treatment of cultured cells (Razin *et al.*, 1984).

Treatment with monoclonal antibodies and complement

IL-3-dependent cells and splenocytes were incubated at the concentration of 10^7 cells/0·5 ml with monoclonal anti-Thy 1.2 antibodies (lot FPB-222, NEN) diluted 1:200, or Lyt 1.1 (Lot FBS-045, NEN) and Lyt 2.1 (Lot FPA-358, NEN), both diluted 1:20. In order to kill antibody-coated cells, low toxicity rabbit C (Low-Tox M, Cedarlane Laboratories, Hornby, Ontario, Canada) was employed at a final dilution of 1:14. Cells were incubated at 4° for 30 min with the above mentioned antibodies, then washed once and resuspended in diluted C for 45 min at 37°. Cells were then washed twice and resuspended in medium without readjusting the cell concentration.

Cytotoxicity assays

The target cells employed were WEHI-164 obtained from Dr A. Mantovani, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy; YAC-1, obtained from Dr R. Kiessling, Karolinska Institutet, Stockholm, Sweden; P815, obtained from Dr D. Collavo, Università di Padova, Padova, Italy; Meth A, obtained from Dr G. Forni, Università di Torino, Torino, Italy. All these lines were maintained in complete medium in exponential growth. Effector cells were IL-3-cultured cells or splenocytes and lymphocytes from Peyer's patches obtained by gentle teasing of these organs. Tumour cells suspended in 1 ml of complete medium were incubated for 90 min at 37° with 100 μCi of Na_2 $^{51}CrO_4$ (The Radiochemical Centre, Amersham, Bucks, U.K.). Then, 10^4 washed cells were incubated with lymphoid cells for 4 and 20 hr at varius effector to target (E:T) ratios in 0·6-cm round bottom wells (Cat. no. 76-022-05, Linbro, Hamden, CT). The percentage of isotope release was calculated from the formula:

$$\% \text{ specific cytotoxicity} = \frac{\text{c.p.m. released from experimental group} - \text{c.p.m. spontaneous release}}{\text{total releasable c.p.m.} - \text{c.p.m. spontaneous release}} \times 100.$$

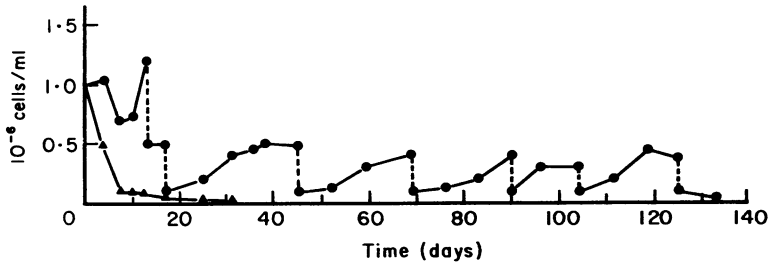

Figure 1. Growth kinetics of bone marrow cells in the presence (●——●) or absence (▲——▲) of IL-3. Broken lines represent culture splittings.

Spontaneous release was 10–20% in the 4-hr assay and 25–30% in the 20-hr assay. Experimental groups were tested in triplicate.

RESULTS

Cells from mouse bone marrow can differentiate and grow in the presence of IL-3, whereas they survive without replicating for only 2–4 weeks if cultured without IL-3. As shown in Fig. 1, the optimal *in vitro* growth of IL-3-dependent bone marrow cells was observed to be from 10^5 to 5×10^5 cells/ml with a doubling time of about 4–5 days. These cultures could usually be maintained for up to 3–4 months. After the first 2 weeks *in vitro*, the majority of the cells showed a morphology similar to that of mast cells. As shown in the inset of Fig. 2, IL-3-dependent cells contained a single nucleus, and a heterogeneous population of granules stained metachromatically with toluidine blue. Moreover, the mast cell-like morphology of IL-3-dependent cells was confirmed by transmission

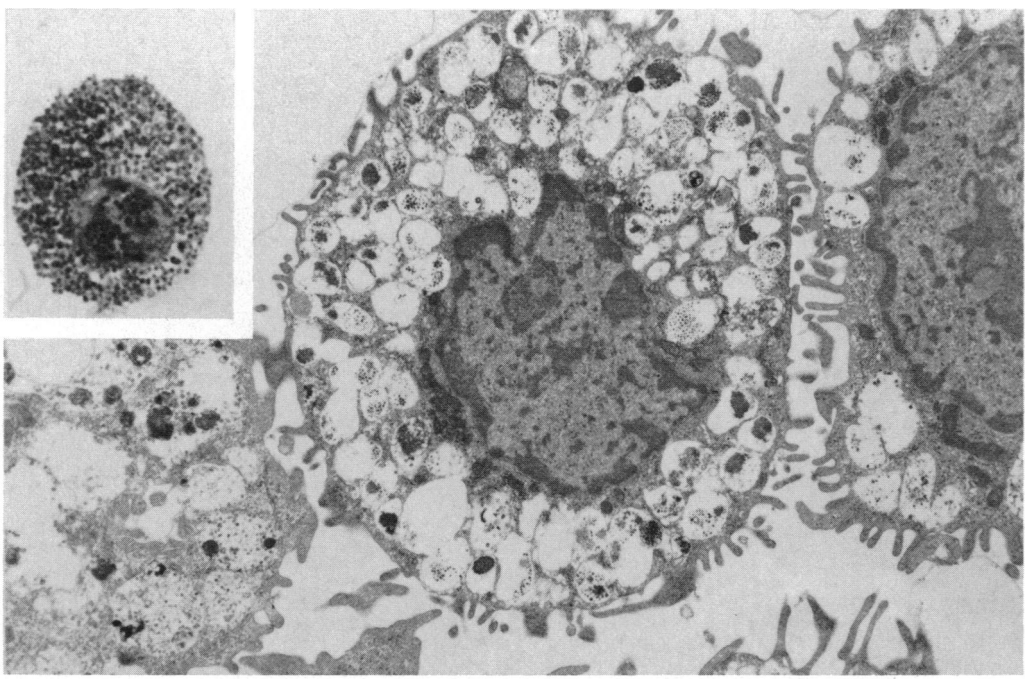

Figure 2. Morphology of IL-3-dependent cells by optical (inset: toluidine blue staining at pH 3, magnification ×2520) and transmission electron microscopy (magnification ×6300).

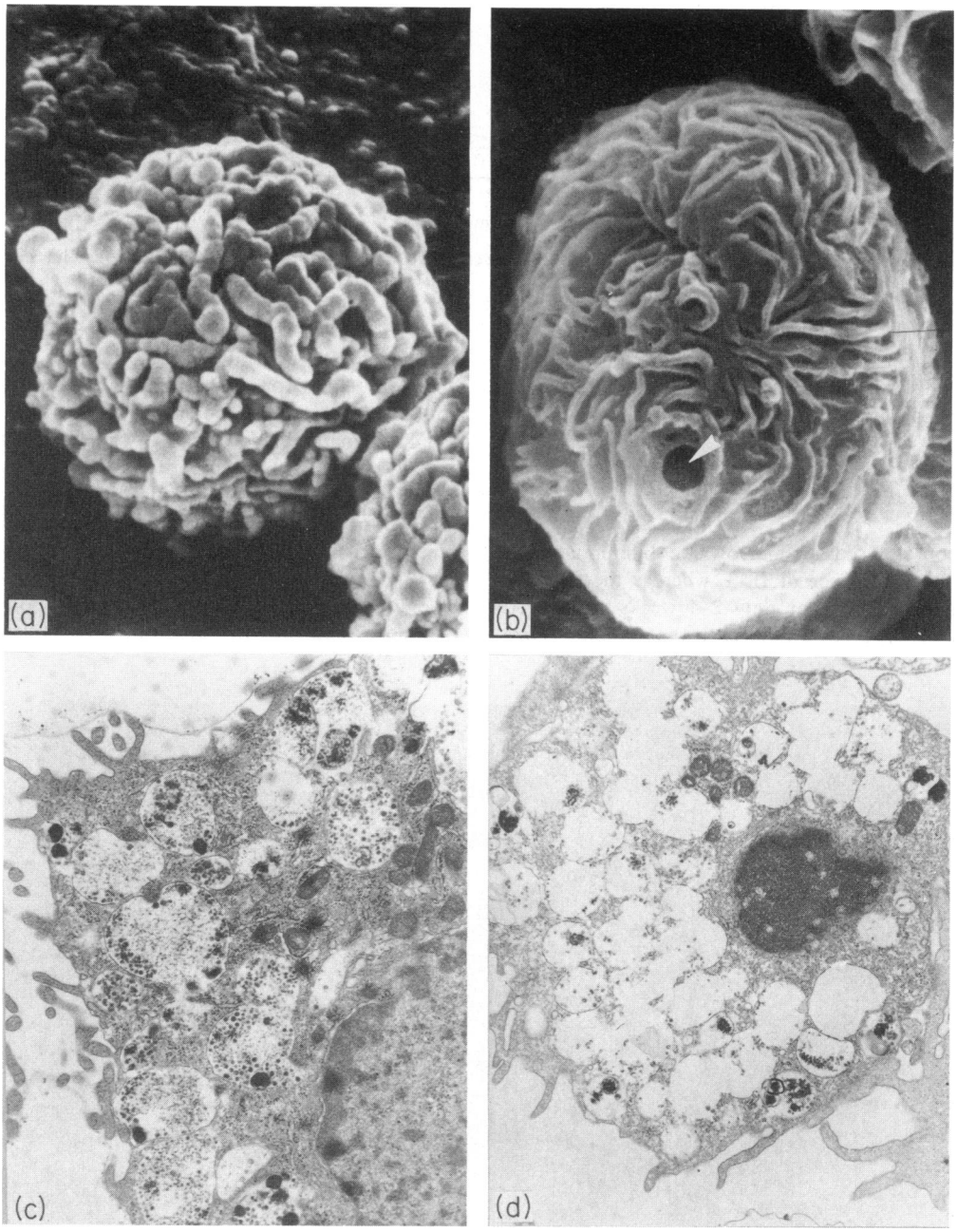

Figure 3. Morphology of IL-3-dependent cells by SEM (a) before (magnification ×8100) and (b) after (magnification ×7200) stimulation. Ultrastructure of cytoplasmic granules of IL-3-dependent cells observed by TEM (c) before (magnification ×10,800) and (d) after (magnification ×9000) stimulation.

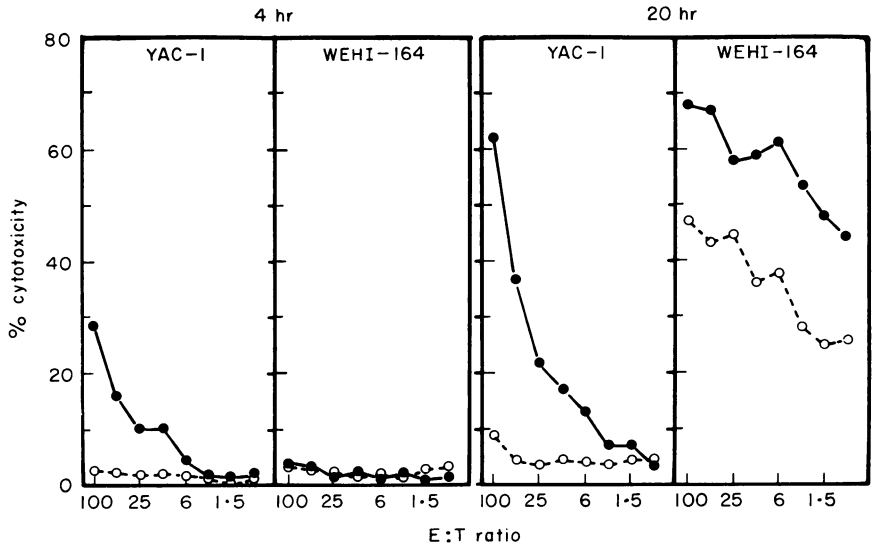

Figure 4. Cytotoxicity of IL-3-dependent cells (O- - -O) and fresh splenocytes (●——●) against YAC-1 and WEHI-164 tumour cells in 4-hr and 20-hr ^{51}Cr-release assays.

electron microscopy (Fig. 2). In fact, as previously reported by others (Galli et al. 1982b; Razin *et al.*, 1984), these cells showed a cytoplasm filled by secretory granules constituted by a light matrix containing one or more dense cores of 0·2 μm diameter, vesicular membrane fragments and interspersed fibrillar material (Figs 2 and 3c), indicating an early stage of differentiation. When IL-3-dependent cells were treated with IgE/anti-IgE complexes, their morphology closely resembled that of stimulated mast cells (Henderson *et al.*, 1981). In fact, on SEM observation, the surface is normally constituted by irregularly arranged foldings (Fig. 3a). After treatment, it showed thinner, more regularly distributed foldings with the concomitant appearance of characteristic pores (Fig. 3b, arrow). At TEM observation, the cytoplasmic granules of treated cells appeared empty and mostly fused (Fig. 3d), indicating degranulation. Biochemical evidence further indicated that the IL-3-dependent cells are similar to immature mast cells. In fact, these cells contained low levels of histamine (65 ng/10^6 cells) and they were induced to release LTC_4 (25·2 ng/10^6 cells) by treatment with IgE/anti-IgE.

The IL-3-dependent mast cells (IL-3 MC) were then used as effectors in cytotoxicity assays against tumour cells. Figure 4 shows a representative experiment of the eight performed, in which a population containing 99% IL-3 MC cultured for 35 days was tested in parallel to splenocytes for its activity against YAC-1 and WEHI-164 tumour cells, i.e. the optimal targets for NK and NC activities, respectively. It is shown that IL-3 MC do not possess NK activity, whereas they can express a significant NC activity in a 20-hr assay. In further support of the capability of IL-3 MC to exert specifically NC activity, it was found that they could kill Meth A tumour cells in a 20-hr assay (Lattime *et al.*, 1983) but not P815 mastocytoma cells, i.e. the target of the recently described 'aged killer activity' (Simon *et al.*, 1984) (Table 1). A preliminary characterization of the phenotype of the effector cells of NC

Table 1. Cytotoxicity of IL-3-dependent mast cells against different tumour targets

Exp. no.	Target	4-hr assay		20-hr assay	
		IL-3 MC	Spleen	IL-3 MC	Spleen
2	WEHI-164	3·6*	3·4	29·6	28·3
	Meth A	4·9	3·5	10·6	13·3
	YAC-1	<1	16·9	<1	20·6
8	WEHI-164	<1	1·0	17·1	44·4
	YAC-1	<1	17·6	<1	35·2
	P815	1·0	3·8	<1	3·4

* Percent specific cytotoxicity at the E:T ratio of 25:1.

Table 2. Characterization of the phenotype of the IL-3 mast cell exerting NC activity

Cell source	Treatment	Target	
		YAC-1	WEHI-164
Fresh spleen*	None	31·2†	26·3
	C	25·1	17·0
	Anti Thy 1.2+C	10·6‡	20·2
	Anti Lyt 1.1+C	19·0	20·0
	Anti Lyt 2.1+C	27·7	21·1
IL-3-dependent bone marrow 23-day cultures§	None	<1	36·1
	C	<1	34·0
	Anti Thy 1.2+C	<1	30·2
	Anti Lyt 1.1+C	<1	37·6
	Anti Lyt 2.1+C	<1	40·7

* E:T ratio = 60:1.
† Percent specific cytotoxicity in a 20-hr ^{51}Cr-release assay.
‡ $P \leqslant 0{\cdot}05$ versus C control assessed by Student's *t*-test.
§ E:T ratio = 20:1.

activity among IL-3 MC revealed similarities with the phenotype of the splenic NC effector. In fact, the treatment with monoclonal antibodies against Thy 1.2, Lyt 1.1 and Lyt 2.1 did not reduce the NC activity of either splenocytes or IL-3 MC in the presence of C (Table 2).

Finally, in an attempt to establish whether NC activity can also be expressed *in vivo* by cells of the mast cell lineage, we tested W/W^v mice, previously reported for their deficiency in mast cell precursors (Kitamura, Go & Hatanaka, 1978), for their *in vitro* activity against WEHI-164 tumour cells. As shown in Table 3, no difference in the NC expression by splenocytes and cells from Peyer's patches, two anatomical districts where NC is highly expressed (Tagliabue *et al.*, 1984), was observed between W/W^v deficient mice and their normal littermates.

Table 3. Natural cytotoxicity of W/W^v mice against WEHI-164 tumour cells

Cell source	E:T ratio	Mouse strain			
		W/W^v	W^v/+	W/+	+/+
Spleen	50	65·0*	72·7	83·5	83·4
	25	79·0	72·9	78·8	83·4
	12	79·3	61·7	81·9	76·8
	6	65·9	68·0	76·4	73·1
	3	68·2	72·4	71·8	71·1
	1·5	55·4	54·9	67·2	67·3
Peyer's patches	50	66·5	66·9	67·8	61·8
	25	57·3	56·3	61·5	50·0
	12	44·8	43·8	46·2	40·7
	6	24·5	32·8	40·9	26·8
	3	23·6	22·4	29·3	15·9
	1·5	15·6	10·8	16·0	13·1

* Percent specific cytotoxicity in a 20-hr ^{51}Cr-release assay.

DISCUSSION

The concept that the host possesses cells capable of lysing tumours *in vitro* without prior immunization is now established, and the first, but not conclusive, results in support of an *in vivo* role for this natural defence mechanism have begun to appear in the literature (reviewed in Herberman, 1982). However, after a decade since the first description of natural anti-tumour reactivity, the lineage of the cells involved in this immune mechanism remains a matter of debate. An important step towards the clarification of this issue came with the observation by Timonen *et al.* (1979) that lymphocytes with abundant cytoplasm full of azurophilic granules (LGL) are the major effector of NK activity in man. Subsequently, LGL-associated NK activity has been found in mice (Luini *et al.*, 1981) and rats (Reynolds, Timonen & Herberman, 1981), the most employed experimental species, offering the possibility to perform transfer studies in order to verify the *in vivo* role of NK activity. The first requirement for this experimental approach is the availability of high numbers of pure LGL. Since, in nature, LGL are rare and difficult to isolate, many groups have tried to solve the problem by growing LGL in culture with the aid of lymphokines and screening for morphology and/or cytotoxic capabilities. However, it soon became clear that cultured cells with LGL-like morphology do not always function as their normal counterparts, particularly in terms of target specificity (Brooks *et al.*, 1982). Even more paradoxical results came when murine cytotoxic T lymphocytes in culture were tested against different tumours, including NK targets. It appeared that some of them can acquire a reversible NK-like activity (Brooks, 1983), whereas others can switch their killer specificity after a long time in culture, i.e. the so-called 'aged killers' (Simon *et al.*, 1984). It has also been shown that cloned splenic T cells resembling basophils by morphological and biochemical criteria can express

NK activity (Galli *et al.*, 1982a). However, Galli *et al.* (1982b) have also shown that cloned bone-marrow cells resembling immature mast cells do not express NK activity. When another natural resistance mechanism such as NC activity (reviewed in Herberman, 1982) was examined, it was found that IL-3 specifically boosts this activity after overnight treatments (Lattime *et al.*, 1983), and that lymphocytes maintained in culture with this lymphokine continue to express NC activity (Djeu *et al.*, 1983). However, no information about the morphology of the effector cells was provided. Thus, it can be concluded that, in the presence of different growth factors, great flexibility in expressing natural cytotoxic reactivities can be observed.

In an attempt to clarify some of the contradictory results mentioned above, in this study we analysed the cytotoxic capability of bone marrow derived murine mast cells differentiated *in vitro* in the presence of IL-3. In agreement with the previous results of other groups (Galli *et al.*, 1982b; Razin *et al.*, 1984), the bone marrow cells maintained *in vitro* in the presence of IL-3 acquired the characteristics of mast cells in about 2 weeks. In fact, these cells contained a single nucleus and a heterogenous population of granules metachromatically stained with toluidine blue. Furthermore, their ultrastructural characteristics resembled those of immature mast cells. Finally, our cells in culture contained low levels of histamine, and degranulated in the presence of IgE/anti-IgE complexes releasing LTC_4.

When IL-3 MC were tested against tumour targets in short- and long-term cytotoxicity assays, it was found that they can only kill WEHI 164 and Meth A in 20-hr assays, and not YAC-1 or P815. Indeed, the former two tumour lines are thought to be the optimal targets for the NC activity, whereas the latter are the best targets for NK and aged cytotoxicity, respectively. Thus, IL-3 MC express only NC activity and, as previously shown by Galli *et al.* (1982b), they are unable to mediate NK lysis. In order to further support this conclusion, treatment with anti-Thy 1, Lyt 1 and Lyt 2 antibodies and C did not affect IL-3 MC cytotoxicity as previously shown for NC activity by freshly isolated cells (Djeu *et al.*, 1983). This would confirm the previous results of Djeu *et al.* (1983) showing the NC activity of cells cultivated in IL-3, and extending them with the observation that mast cells are the effectors of this anti-tumour activity. Since the effector cell of NC activity has not been positively identified, it might be suggested that the data obtained with *in vitro* cultured mast cells would indicate that cells of this lineage are also the NC effectors *in vivo*. Indeed, previous results have shown that freshly isolated peritoneal mast cells can kill tumours *in vitro*, even though no NC targets were employed in these studies (Farram & Nelson, 1980; Henderson *et al.*, 1981). In an attempt to prove this hypothesis, we tested mast cell-deficient W/W^v mice (Kitamura *et al.*, 1978) and their normal littermates, employing as source of effector cells two organs, spleen and Peyer's patches, known for the presence of high NC activity (Tagliabue *et al.*, 1984). Since no decrease in NC activity was observed in W/W^v mice, it seems unlikely that mast cells also play a role *in vivo* in the expression of NC activity, even though the scanty knowledge of the biology of NC activity renders it still possible to hypothesize that a second population of NC effectors is increased in W/W^v mice to replace the mast cell deficiency. In turn, a more general explanation for the above-mentioned acquisition of cytotoxic capabilities in culture by different cell populations may be advanced on the basis of recent results that the cytoplasmic granules of many cell types are an important part of their lytic machinery (Henkart *et al.*, 1984; Podack & Konigsberg, 1984). Thus, the acquisition of granules in culture would provide the lytic capabilities, regardless of the cell lineage, whereas the characteristic surface molecules of the effector cells would ensure their specificity for some tumour targets, as observed here and in other studies. Experiments are currently being performed to prove this hypothesis.

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