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A comparison of membrane markers on rat cytotoxic cells

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Summary. The antigenic characteristics of cytotoxic T cells (CTL) and cells mediating antibody-dependent cellular cytotoxicity (ADCC) were defined using monoclonal antibodies W3/13, W3/25 and MRC OX8, and compared with the phenotype of natural killer (NK) cells previously defined by these antibodies. CTL, ADCC effector cells and NK cells were also tested for expression of Ia antigen using MRC OX6 monoclonal antibody. The fluorescence-activated cell sorter was used to separate effector populations into antigen-positive and antigen-negative subsets, and the cytotoxicity of the resultant lymphocyte fractions was then assessed using a 6 hr ^{51}Cr release assay and a quantitative method of analysis based on consideration of target cell lysis as an enzyme substrate reaction.

CTL, ADCC effector cells and NK cells were W3/25 negative and OX6 negative. CTL were positive for W3/13 and OX8 antibody with no cytotoxicity in the W3/13 negative and OX8 negative fractions. In contrast, ADCC effector cells showed heterogeneity of staining with W3/13 and OX8 antibodies, with 50% cytotoxic activity in the W3/13 negative fraction, and 20–30% cytotoxic activity in the OX8 negative fraction.

In parallel experiments, NK cells and ADCC effector cells showed identical marker heterogeneity when tested with W3/13 and OX8 antibodies.

Abbreviations: ADCC, antibody dependent cellular cytotoxicity; CTL, cytotoxic T lymphocytes; FACS, fluorescence activated cell sorter; FCS, foetal calf serum; HBSS, Hanks's balanced salt solution; 2ME, 2-mercaptoethanol; MEM, minimal essential medium; MLR, mixed lymphocyte reaction; NBCS, newborn calf serum; NK, natural killer; RCA, relative cytotoxic activity.

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INTRODUCTION

The hybridoma technology described by Kohler & Milstein (1975) has been utilized to generate four monoclonal antibodies (W3/13, W3/25, MRC OX6 and MRC OX8) that recognize determinants selectively expressed on rat lymphocyte subpopulations (Williams, Galfre & Milstein, 1977; McMaster & Williams, 1979; Brideau *et al.*, 1980). These reagents are being used extensively in the elucidation of immune mechanisms and are proving useful as markers for functionally distinct lymphocyte subsets (Loop, Bernstein & Wright, 1980; Fernandez-Cruz, Woda & Feldman, 1980; Mason, Pugh & Webb, 1981).

W3/13 is a pan T-cell marker, although it also stains cells other than lymphocytes, including granulocyte precursors. W3/25 and OX8 stain non overlapping T-cell populations with helper/inducer and suppressor/cytotoxic functions respectively. OX6 reacts with a non-polymorphic determinant of rat Ia.

Recently it was shown that rat natural killer (NK) cells and cytotoxic T cells (CTL) label with OX8 and

W3/13 monoclonal antibodies (Woan, McGregor & Goldschneider, 1981; Cantrell *et al.*, 1982). In particular, it was established that rat NK cells are a heterogeneous population, with at least four subsets clearly defined on the basis of surface antigen expression. This NK-cell heterogeneity was readily demonstrable because a quantitative method was employed to analyse NK cytotoxicity. However, this type of analysis has not so far been applied to studies of T-cell cytotoxicity, so it is not yet known whether the T-killer population in the rat is homogeneous or heterogeneous with respect to cell surface markers.

To clarify this point, the present study was initiated. A comparison of the phenotype of the NK and CTL populations was made by using a quantitative approach to evaluate the cytolytic activity of the various lymphocyte subpopulations. Additionally, the cell surface phenotype of the cells mediating antibody-dependent cytotoxicity (ADCC) was examined in an attempt to assess the relationship of this effector population to the NK-effector subset.

In this regard it is interesting that it is not yet known whether NK and ADCC activity are mediated by different cells or whether the same cell is capable of functioning through both antibody-dependent and antibody-independent lytic mechanisms (Ojo & Wigzell, 1978; Roder & Duwe, 1979; Neville, 1980; Fast, Hansen & Newman, 1981; Nabel *et al.*, 1981). Thus, given the marked antigenic heterogeneity of rat NK effectors it was of obvious importance to determine whether a similar heterogeneity was evident in the ADCC-effector population or whether the cells involved in NK and ADCC killing could be dissociated on the basis of surface antigen markers.

MATERIALS AND METHODS

Animals

Inbred WAB/Not, ($RT1^l$) and KX/Not ($RT1^u$) rats and KX/WAB F_1 hybrids were bred in the Cancer Research Campaign Laboratories and used when 2–3 months old. Congenitally athymic rnu/rnu rats were purchased from Olac 1976 Ltd. (Bicester, U.K.).

Preparation of lymphocytes

Minced spleens or lymph nodes (pooled cervical and mesenteric) were gently pressed through 120 gauge screens into Hanks's balanced salt solution (HBSS) containing 1% foetal calf serum (FCS) (Flow Laboratories, U.K.). Red blood cells were lysed by a 5 sec water treatment with sterile distilled water. All cells were washed three times in HBSS + 1% FCS prior to use.

Antibodies

W3/13, W3/25, OX6 and OX8 monoclonal antibodies, raised against rat lymphocytes (Williams *et al.*, 1977; White *et al.*, 1978; McMaster & Williams, 1979; Brideau *et al.*, 1980), were obtained through Seralab (Crawley Down, U.K.). Fluorescein-conjugated rabbit anti-mouse immunoglobulin was obtained from Dako (Mercia Brocades U.K. Ltd). Rat anti-EL4 antiserum was prepared by immunizing WAB/Not rats with 10^7 EL4 cells intraperitoneally once a week for 5 weeks. Thereafter serum was collected, inactivated at 56° for 1 hr and then stored at −70°.

Cell fractionation

Phagocytic cells were removed with a strong magnet following a 1 hr incubation (37°) in Eagles MEM + 10% FCS with 10 mg/ml carbonyl iron (GAF Ltd. Manchester, U.K.) which was washed three times with HBSS + 1% FCS before use. Recovered lymphocytes were washed twice. This procedure has been shown to remove all macrophage cytotoxic activity (Gray, Brooks & Baldwin, 1981), and was used routinely for all cell preparations analysed for NK and ADCC activity.

Cell separations were carried out on a fluorescence-activated cell sorter (FACS IV; Becton Dickinson). Lymphocytes were labelled with monoclonal antibody by incubation with an appropriate saturating concentration (4–8 μg/ml) in HBSS + 1% FCS. After 1 hr at 4° the cells were washed three times and then incubated for 1 hr at 4° with fluorescein conjugated rabbit anti-mouse IgG (1 : 20 dilution). Labelled lymphocytes were then analysed on the FACS IV and sorted into appropriate fluorescence-positive and -negative populations. Sorting was carried out under sterile conditions and sorted fractions collected on ice. Appropriate forward-angle light-scatter gating excluded dead cells. Cells were sorted at a rate of 2000–2500/sec and under these conditions, average yields were 60% and purity ranged from 97–99%.

Mixed lymphocyte cultures

RPMI 1640 containing 5% FCS, 10^{-5} M 2-mercaptoethanol (2ME) was used. 2×10^7 WAB/Not lymph-node cells were cultured with 10^7 irradiated (2000R) KX/WAB F_1 lymph-node cells in 20 ml volumes in 90 mm petri dishes (Sterilin, Middlesex, U.K.) These were then incubated at 37° in 5% CO_2 for 120 hr.

Cytotoxicity assay

This has been described elsewhere (Brooks & Flannery, 1980). Briefly, 5×10^3 ^{51}Cr-labelled target cells were mixed with graded numbers of effector cells in 300 μl Eagles MEM containing 5% FCS in the wells of Microtest II plates (Sterilin, Middlesex, U.K.). After 6 hr at 37° in 5% CO_2, 100 μl of supernatant were removed and transferred to counting tubes. Counts per minute (c.p.m.) were measured in a LKB gamma counter and were corrected for machine background.

The percentage ^{51}Cr release was computed using the formula:

$$\% \text{ Release} = \frac{\text{c.p.m. in supernatant}}{\text{Total c.p.m. in target cells}} \times 100$$

Thus percentage cytotoxicity is given by:

$$\% \text{ Cytotoxicity} = 100 \times \frac{[(\% \text{ release with effector cells}) - (\% \text{ release with medium})]}{100 - (\% \text{ release with medium})}$$

For ADCC activity cytotoxicity was given by:

$$\frac{100 \times [(\% \text{ release with effectors} + \text{antibody}) - (\% \text{ release with effectors} + \text{normal serum})]}{100 - (\% \text{ release with effectors and normal serum})}$$

Analysis of cytotoxicity

This is presented in detail elsewhere (Brooks & Flannery, 1980). When percentage cytotoxicity (up to 25%) is plotted against effector-cell concentrations, the slope of the regression line passing through the origin is directly proportional to the frequency of cytotoxic cells in the effector population. At least three data points were used for the calculation of each slope. For two effector populations, the ratio of slopes is a direct measure of the relative frequency of cytotoxic cells in the two cell populations. The relative cytotoxic activity (RCA) of two cell populations is therefore calculated as $100 \times S_1/S_2$ where S_1 is the slope for the test cells and S_2 the slope for the control cells. The lytic unit recovery in a test population is then given by $y \times$ (RCA), where y is the fractional yield of cells in the test group compared to the control group. Statistical analysis was performed using students' t test. NS $P > 0{\cdot}05$; * $0{\cdot}05 > P > 0{\cdot}01$; ** $0{\cdot}01 > P > 0{\cdot}001$; *** $0{\cdot}001 > P$.

Target cells

Sarcoma Mc7 was induced in a WAB/Not rat by 3-methylcholanthrene and passaged subcutaneously *in vivo* by regular trocar grafts. Hepatoma D192A was induced in a WAB/Not rat by feeding dimethylaminoazobenzene, and was also passaged by trocar graft. *In vitro* variants of a rat spontaneous fibrosarcoma (SP102) were made available by the kind cooperation of Dr C. G. Brooks and Dr E. A. Wayner. Tumour-cell clones were cultured *in vitro* in MEM + 10% NBCS and were subcultured using 0·25% trypsin (Difco, U.K.). Variant clones were selected after screening for sensitivity to rat splenic NK cells (Brooks *et al.*, 1981).

Mouse leukaemia EL4 was a gift from Dr N. Hogg, University College, London, and was maintained in RPMI 1640 + 10% FCS.

Rat myeloma Y3Ag 1.2.3 was a gift from Dr C. Milstein, MRC Laboratory of Molecular Biology, Cambridge, U.K., and was maintained in Dulbecco's modified Eagles medium (Flow Ltd) supplemented with 5% FCS and 100 mM sodium pyruvate.

No antibiotics were used for tumour-cell cultures. Additionally, cultures were tested for mycoplasma infection, the importance of which has been described previously (Brooks, Rees & Leach, 1979).

Con A blasts were prepared by incubating rat spleen cells at 10^6/ml in RPMI 1640 containing 10% FCS, 10^{-5} M 2ME and 5 μg/ml Con A (Sigma Ltd) for 72 hr.

For the ADCC assay, EL4 target cells were labelled for 1 hr at 4° with 1/1000 dilution of heat inactivated rat anti EL4 antiserum.

RESULTS

The phenotype of rat splenic NK and ADCC effector cells

For studies of rat ADCC activity, EL4 tumour cells coated with rat anti-EL4 antiserum were used as target cells. EL4 was a suitable target because it is resistant to lysis by non-activated populations of rat NK cells. This was important because NK and ADCC effectors have a parallel tissue distribution, so that it is difficult to obtain an *in vivo*-derived population containing ADCC killers without that population also containing NK cells.

As in the NK system, splenic ADCC activity was not susceptible to blocking by W3/13, W3/25, OX6 and OX8 monoclonal antibodies (Table 1). Therefore spleen cells were labelled with one of the antibodies, sorted in the FACS, and the resultant fractions tested in parallel in NK and ADCC cytotoxicity assays. The results of typical experiments are shown in Table 2.

Table 1. Effect of monoclonal antibodies on splenic NK and ADCC activity

Spleen population +antibody	NK cytotoxicity*	ADCC cytotoxicity†
Untreated	$3{\cdot}73 \pm 0{\cdot}13$	$9{\cdot}36 \pm 0{\cdot}10$
W3/13‡	$4{\cdot}27 \pm 0{\cdot}37^{NS}$	$9{\cdot}28 \pm 0{\cdot}67^{NS}$
W3/25‡	$3{\cdot}77 \pm 0{\cdot}46^{NS}$	$8{\cdot}77 \pm 0{\cdot}45^{NS}$
OX6	$3{\cdot}93 \pm 1{\cdot}06^{NS}$	$8{\cdot}85 \pm 1{\cdot}02^{NS}$
OX8‡	$4{\cdot}38 \pm 0{\cdot}06^{NS}$	$9{\cdot}41 \pm 0{\cdot}69^{NS}$

* Slope $\pm$ SD $\times 10^{-5}$ of the regression line for cytotoxicity; sarcoma Mc7 as target cells.

† Slope $\pm$ SD $\times 10^{-5}$ of the regression line for cytotoxicity; antibody-coated EL4 cells as targets.

‡ >1 μg/ml.

Both NK and ADCC effectors are W3/25− and OX6− and both show similar heterogeneity with regard to the antigens defined by W3/13 and OX8 antibodies (60–70% OX8+; 20–30% OX8−; 50% W3/13+; 50% W3/13+). Despite the clear heterogeneity of rat spontaneous effector cells, NK and ADCC cytotoxicity always separated in parallel and there was no evidence that these two types of cytolytic activity could be dissociated using W3/13, W3/25, OX6 and OX8 antibodies.

The surface phenotype of rat CTL

Cytolytic lymphocytes were generated in a MLR using KX or WAB rat lymph-node cells as responders and irradiated KX/WAB F_1 lymph-node cells as stimulators. The specificity of these effector cells was examined using a range of NK-susceptible and -resistant target cells. At the same time the cytotoxicity of an NK population (normal spleen cells) against this target-cell panel was assessed. It is clear from the data (Table 3) that the MLR killer cells show a specificity distinct from that of the NK population. Thus they show preferential killing of target cells which share the haplotype of the determinants on the stimulator lymphocyte population, regardless of the NK susceptibility or resistance of that target. Only KX anti-WAB killers lyse WAB targets and only WAB anti-KX killers lyse KX targets. This type of specificity defines these particular MLR effector cells as CTL.

Table 2. The phenotype of rat splenic ADCC effector cells

Cell fractionation	Cytotoxic activity* (slope $\pm$ SD $\times 10^{-5}$)	RCA†	Yield	Lytic unit recovery ADCC§	Lytic unit recovery NK¶
Unfractionated	$13{\cdot}07 \pm 0{\cdot}19$				
W3/25−	$22{\cdot}45 \pm 0{\cdot}31$	171	55	94	92
W3/25+	$0{\cdot}18 \pm 0{\cdot}58$	1·4	40	0·6	1
Unfractionated	$9{\cdot}36 \pm 0{\cdot}10$				
OX6−	$25{\cdot}51 \pm 1{\cdot}07$	272	42	114	93
OX6+	$1{\cdot}16 \pm 0{\cdot}43$	12	49	5	3
Unfractionated	$14{\cdot}01 \pm 1{\cdot}24$				
W3/13−	$31{\cdot}46 \pm 3{\cdot}61$	225	25	56	50
W3/13+	$10{\cdot}88 \pm 0{\cdot}51$	77	75	57	41
Unfractionated	$10{\cdot}77 \pm 0{\cdot}57$				
OX8−	$3{\cdot}71 \pm 0{\cdot}32$	34	70	23	28
OX8+	$27{\cdot}65 \pm 1{\cdot}08$	257	26	71	66

* EL4 coated with rat anti-EL4 antiserum as targets.

† Relative cytotoxic activity, % relative to unfractionated cells.

‡ Percentage of stained/non stained cells.

§ Product of yield + RCA for ADCC target.

¶ NK cytotoxicity was also assessed. Only lytic unit recovery data is shown. The NK target was sarcoma Mc7.

Table 3. The specificity of cytotoxic cells generated in the rat mixed lymphocyte reaction

Target cell	Haplotype	Cytotoxicity (slope ± SD × 10^{-5}) NK*	KX anti-WAB† T killers	WAB anti-KX‡ T killers
Blasts	WAB	2·74 ± 0·39 −	15·24 ± 0·57 +	ND
Blasts	KX	0·91 ± 0·26 −	0·73 ± 1·92 +	12·48 ± 0·19 +
Mc7	WAB	6·83 ± 0·46 +	13·87 ± 1·05 +	1·89 ± 0·67 −
Y3Ag 1.2.3	KX	16·53 ± 0·98 +	1·43 ± 0·97 −	11·07 ± 0·31 +
SP102C	WAB	3·92 ± 0·38 +	3·24 ± 0·11 +	0·98 ± 0·21 −
SP102B	WAB	2·26 ± 0·55 −	11·62 ± 0·15 +	1·98 ± 0·23 −
SP102f	WAB	0·83 ± 0·21 −	9·69 ± 0·74 +	0·59 ± 0·13 −
D192A	WAB	1·69 ± 0·25 −	8·52 ± 0·09 +	2·47 ± 0·43 −

* Normal splenic NK effectors.

† KX anti-WAB T killers were generated using KX lymph node cells as responders and WAB lymph-node cells (irradiated) as stimulators (120 hr culture).

‡ WAB anti-KX T killers were generated using WAB lymph-node cells as responders and KX lymph-node cells (irradiated) as stimulators (120 hr culture).

− Relatively resistant target (slope below 3·00).

\+ Relatively susceptible target (slope above 3·00).

The antigenic phenotype of the rat CTL population was then determined. Effector cells were labelled with one of four monoclonal antibodies and then sorted in the FACS. The data (Table 4) show that no significant cytotoxicity was mediated by W3/25+, OX6, W3/13− and OX8− cells, and, by taking into account lytic units, it is clear that the majority of rat CTL activity is associated with lymphocytes that express antigens defined by W3/13 and OX8 but not W3/25 and OX6 monoclonal antibodies. Neither W3/13 nor

Table 4. The phenotype of cytolytic T cells generated in the rat mixed lymphocyte reaction

Cell population	Cytotoxicity* (slope ± SD × 10^{-5})	RCA†	Yield	Lytic unit recovery
Unfractionated	1·38 ± 0·08			
OX6−	3·88 ± 0·23	281	50	140
OX6+	0·08 ± 0·16	5·7	45	2
Unfractionated	2·48 ± 0·21			
W3/25−	5·79 ± 0·14	233	47	109
W3/25+	0·18 ± 0·04	7	48	3·5
Unfractionated	1·53 ± 0·06			
W3/13−	0·07 ± 0·8	5	37	2
W3/13+	2·63 ± 0·31	171	60	102
Unfractionated	3·66 ± 0·26			
OX8−	0·09 ± 0·23	2·5	50	1
OX8+	9·18 ± 1·5	251	40	100

* WAB anti-KX lymph-node T killer cells with Y3Ag 1.2.3 as target cells. Effectors were generated in a 120 hr MLR culture.

† Relative cytotoxic activity, % relative to unfractionated cells.

OX8 antibodies had any blocking activity on T-cell mediated lysis (data not shown).

DISCUSSION

In the present study, the antigen expression of rat CTL, NK and ADCC effectors was examined. The cytotoxicity of lymphocytes fractionated according to their surface markers was quantified using a short term ^{51}Cr-release assay, and the activity of the various subsets was then evaluated in terms of the total lytic activity of the original effector population.

It is absolutely essential to apply this form of analysis to studies of the cytotoxicity of lymphocyte subpopulations. This is perhaps emphasized by the studies of Gray *et al.* (1981), who showed that only by employing lytic unit analysis could it be demonstrated that BCG induces both macrophage and NK effectors when given intraperitoneally to either nude or normal rats. Additionally, it is important in fractionation studies to use both negative and positive selection techniques. Only by the use of the latter can direct evidence be obtained for the expression of a surface marker by a particular subset of cytolytic effector cells.

Using positive selection and quantitative methods of analysis it was demonstrated that rat CTL, and subpopulations of NK and ADCC effector cells share common antigenic determinants defined by W3/13 and OX8 monoclonal antibodies. These two surface structures did not appear to be involved in the cytolytic or recognitive machinery of the cells analysed, since neither antibody had any blocking activity in the CTL, NK or ADCC assays.

None of the cytolytic effector cells examined labelled with W3/25 or OX6 antibodies. OX6 recognizes rat Ia antigens and thus, in contrast to previous reports (Duarte, Carpenter & Strom, 1982), the present data suggest that cytotoxic T cells do not express Ia antigens. This discrepancy can perhaps be explained by observations that the expression of Ia antigens on CTL is variable and appears to be related to the state of cell activation. In humans, CTL from day 7, but not day 5 MLR cultures, express Ia antigens (Fast *et al.*, 1981). In the present study, day 5 MLR effectors were analysed, so it is quite feasible that the examination of effectors from subsequent time points would yield different results.

The finding from the present study that rat NK cells label with W3/13 and OX8 correlates well with studies showing that large granular rat lymphocytes also label with these two antibodies (Reynolds *et al.*, 1981b). The significance of this latter study is that Reynolds, Timonen & Herberman (1981a) have suggested, but by no means proved, that the NK population and large granular lymphocyte populations are synonymous and completely overlapping in the rat.

Originally, W3/13 and OX8 antibodies were described as specific T-cell associated markers (Williams *et al.*, 1977; Brideau *et al.*, 1980). W3/13 is a pan T-cell marker, whereas OX8 appears to mark the rat cytotoxic/suppressor T-cell subset analogous to that defined by Ly 23 antisera in mice and the OKT8 antibody in humans (Brideau *et al.*, 1980). However, the present study shows that this analogy is incorrect, particularly with regard to any parallels between OX8, Ly 23 and OKT8. These last two markers do not label the NK effector populations (Koo *et al.*, 1980; Zarling & Kung, 1980). Moreover, whereas OX8 has no blocking effects on rat cytotoxic cells, both OKT8 and Ly 23 antibodies have been reported to block cytotoxicity when used as non-lytic reagents (Hollander, Pillemer & Weissman, 1980; Reinherz *et al.*, 1981).

The finding that rat NK cells and T cells share certain common surface markers is consistent with previous studies of NK effectors in mice and humans. Thus the classic T-cell markers Thy 1 and SRBC receptors are found on the murine and human NK populations respectively (Koo *et al.*, 1980; Fast *et al.*, 1981). However, it is essential that data showing shared antigenic determinants not be used as evidence that T cells and NK cells share a common lineage. It is becoming increasingly clear that there are few surface markers specific for cells of a particular lineage. The definition of a T-cell population must rest on a requirement for thymic processing or maturation, and on these grounds it is clear thar markers such as Thy 1 in the mouse, and indeed W3/13 and OX8 in the rat, are not restricted to T cells since all three label cells in congenitally athymic animals (Brideau *et al.*, 1980). By similar reasoning it is clear that NK cells are not mature T cells, since they also do not require thymic processing to acquire full functional reactivity (Herberman & Holden, 1978).

Marked heterogeneity among NK cells has previously been demonstrated using the W3/13 and OX8 antibodies (Cantrell *et al.*, 1982). We now report similar surface marker heterogeneity among ADCC effector cells, but interestingly not in the CTL population. NK and ADCC effector cells have an almost identical distribution of W3/13 and OX8 markers, which means that these two independent monoclonal

antibodies cannot be used individually to dissociate NK and ADCC cytotoxicity. This surely suggests a close relationship between the rat effector cells involved in these two distinct forms of cytolytic activity. However, it is not a finding that provides conclusive evidence that a single cell or cell population is responsible for both antibody-dependent and antibody-independent spontaneous cytotoxicity.

Indeed this latter point is the subject of many conflicting reports. Fast *et al.*, (1981) have shown that human NK and ADCC effectors have an identical surface phenotype and there is also evidence that single cell-derived NK cell clones can mediate both NK and ADCC cytotoxicity (Nabel *et al.*, 1981). However, other studies have shown that the NK and ADCC effector cells differ with respect to surface Fc receptors (Neville, 1980), and dissociation of NK cell and ADCC activity is noted in certain immunodeficiency states (Koren, Amos & Buckley, 1978).

Thus the relationship between NK and ADCC effector cells is a controversial issue and it is often difficult to reconcile the many discrepant findings. No doubt many differences reflect differences in the target cells employed, species differences and differences in antibodies used for the ADCC assay. Also there is no doubt that the issue is clouded by the marked heterogeneity of the NK population.

In view of this emerging concept of NK-cell heterogeneity, many of the early studies on NK and ADCC effectors may require re-interpretation. Obviously, further analysis of cloned lines of cytotoxic cells is required, as is analysis of NK and ADCC killing at the level of interaction between a single effector and two different target cells. This latter approach is the only one which can directly solve the question of uni- versus multi-potentiality of effector cells (Bradley & Bonavida, 1982).

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