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Phenotype of rat natural killer cells defined by monoclonal antibodies marking rat lymphocyte subsets

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Summary. The fluorescence-activated cell sorter and a rosette-depletion technique were used to separate rat spleen lymphocytes and BCG-activated lymphocytes into subpopulations with and without the antigens defined by W3/13, W3/25 and OX8 monoclonal antibodies. The resultant populations were then tested for natural killer (NK) activity in a quantifiable 6 hr ^{51}Cr release assay. The data establish that rat natural killer cells are heterogeneous with respect to their surface antigen expression and that subpopulations of rat NK cells express the OX8 and W3/13 defined antigens. However, rat NK cells do not express the antigen defined by W3/25 monoclonal antibody.

INTRODUCTION

The hybridoma technique described by Kohler & Milstein (1975) has been employed to produce monoclonal antibodies that react with cell surface determinants expressed selectively on lymphocyte subpopulations. In the rat, three monoclonal antibodies that label T-lymphocyte populations have been described; W3/13 which labels all T cells (Williams, Galfre & Milstein, 1977) and MRC OX8 and W3/25 which label non-overlapping T-cell subpopulations (Brideau, Carter, McMaster, Mason & Williams, 1980; White, Mason, Williams, Galfrie & Milstein, 1978).

These monoclonal antibodies are being used extensively to investigate cellular aspects of the immune response in rats (Loop, Bernstein & Wright, 1980; Fernandez-Cruz, Woda & Feldman, 1980; Barclay, 1981) and are proving useful as markers for functionally distinct subpopulations of lymphocytes. Indeed, it has been proposed (Brideau *et al.*, 1980) that W3/25 and OX8 define lymphocyte subsets in the rat analogous to those defined by the Ly series of alloantisera in mice (Cantor & Boyse, 1975). Thus W3/25 labels the rat helper T-cell subset (Ly 2^-3^-, abundant Ly1 in mice) functional in mixed lymphocyte reactions, graft versus host reactions and antibody responses (White *et al.*, 1978; Webb, Mason & Williams, 1979). While MRC OX8 labels the rat mixed lymphocyte reaction generated cytotoxic T cells (Ly 2^+3^+ in mice) and also the cell population responsible for allogeneic suppression (Brideau *et al.*, 1980; Dallman & Mason, personal communication).

However, it is perhaps important to note that neither W3/25 nor OX8, or indeed W3/13, are res-

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Abbreviations: BSS, balanced salt solution; BCG, Bacillus Calmette-Guerin; FACS, fluorescence-activated cell sorter; FCS, foetal calf serum; MEM, Eagle's minimum essential medium; NK cells, natural killer cells; RCA, relative cytotoxic activity; SRBC, sheep red blood cells; PBS, phosphate-buffered saline.

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tricted entirely to mature T cells. All three monoclonal antibodies label spleen cells in nude rats (Brooks, Webb, Robins, Robinson, Baldwin & Festing, 1980b; Brideau *et al.*, 1980). Additionally, W3/25 labels macrophages (Barclay, 1981) and W3/13 labels polymorph and granulocyte precursors in bone marrow (Williams, Galfre & Milstein, 1977).

Clearly then, although W3/13, W3/25 and OX8 are useful as lymphocyte markers in the rat, they cannot be used as definitive T-cell markers and it is obviously necessary for the cell populations labelled by these monoclonal antibodies to be more thoroughly examined for functional activity.

Recently, there has been much interest in cell populations mediating natural cytotoxicity (Kiessling & Wigzell, 1979). However, it has not been established whether W3/13, W3/25 and OX8 label such spontaneous effector cells in the rat, even though these monoclonal antibodies have been used to characterize effector cells in tumour systems where natural killer (NK) cells may play an important role (Fernandez-Cruz *et al.*, 1980).

To clarify this point, we initiated an investigation to determine whether resident splenic NK cells or Bacillus Calmette-Guerin (BCG)-activated NK cells labelled with W3/13, W3/25 or MRC OX8 monoclonal antibodies. The results indicate that rat NK cells are heterogeneous with respect to their surface markers and that they label with OX8 and W3/13, but not W3/25 monoclonal antibodies.

MATERIALS AND METHODS

Animals

Inbred WAB/Not rats ($RT1^1$) were bred in the Cancer Research Campaign Laboratories and used when 2–3 months old.

Tumours

Sarcoma Mc7 was induced with 3-methycholanthrene and has been transplanted in syngeneic WAB/Not rats. Mc7 cells were cultured in Eagle's minimum essential medium (MEM) + 10% new born calf serum (Flow Laboratories) without antibiotics, and were subcultured using 0·25% trypsin (Difco).

Antibodies

W3/25, W3/13 and OX8 monoclonal antibodies, raised against rat lymphocytes (Williams *et al.*, 1977, White *et al.*, 1978, Brideau *et al.*, 1980) were obtained through Seralab (U.K.). Fluorescein-conjugated rabbit anti-mouse immunoglobulin was obtained from Nordic Immunological Laboratories. Immunoabsorbed purified rabbit anti-mouse IgG $F(ab')_2$ was prepared by ammonium sulphate precipitation, pepsin digestion, fractionation on a Sephadex G-200 column and finally by affinity chromatography using Sepharose 4B coupled to mouse IgG.

Antibody labelling

Lymphocytes were incubated for 1 hr at 4° with a saturating concentration of monoclonal antibody (4–8 μg/ml in Hanks's balanced salt solution). After three washes the cells were incubated for 1 hr at 4° with fluorescein-conjugated rabbit anti-mouse IgG (1:20 dilution).

Coupling of antibody to sheep red blood cells

Two hundred and fifty microlitres of packed sheep red blood cells (SRBC) washed three times with saline, 200 μl antibody [250 μg affinity purified $F(ab')_2$ of rabbit anti-mouse IgG] and 5 ml of saline were mixed with 200 μl of 0·1% chromic chloride. These SRBC were washed twice in phosphate-buffered saline (PBS) and once in Hanks's BSS/2% foetal calf serum (FCS) and then resuspended in 5 ml Hanks's BSS/2% FCS.

Effector cells and cell fractionation

Spleens were removed from WAB/Not rats, pressed through 120 gauge stainless steel screens and washed twice in Hanks's BSS before resuspension in either Hanks's BSS/2% FCS or MEM/10% FCS. Red cells were lysed by a five second water treatment. Phagocytic cells were removed with a strong magnet following a 1 hr incubation (37°) in MEM/10% FCS with 10 mg/ml carbonyl iron (GAF Ltd, Manchester, washed three times with Hanks's BSS before use). Recovered spleen cells were washed once. This treatment has been shown to remove macrophage helper, suppressor and cytotoxic activity (Gray & Brooks, 1980). BCG-activated NK cells were obtained from the peritoneal exudate cells of rats injected intraperitoneally with 0·1 mg moist weight of BCG (BCG percutaneous vaccine, Glaxo Laboratories, Greenford). Cells were washed twice in Hanks's BSS/2% FCS and then carbonyl iron treated to remove cytotoxic macrophages (Gray, Brooks & Baldwin, 1981). Rosette depletions were carried out according to Parish & Hayward (1974) with modifications developed by Dr D. W. Mason (personal communication). Labelled lymphocytes at 10^8/ml were mixed with an equal volume of SRBC

coated with rabbit anti-mouse IgG $F(ab')_2$. Bottles were topped up with Hanks's BSS 2% FCS and then rotated for 20 min at 4°. They were then placed on ice for 5–10 min, centrifuged at 200 *g* and the supernatant recovered. This procedure was repeated. The supernatants from both collections consisted of non-rosetted lymphocytes and SRBC the latter were lysed by a five second water treatment. With this technique yields of non-rosetted lymphocytes were approximately 70%–80% of theoretical. Purity of depleted populations was checked by analysis on the fluorescence-activated cell sorter (FACS) and was usually greater than 99% (Fig. 1); FACS analysis and separations were carried out on the FACS IV (Becton Dickinson). Labelled lymphocytes were analysed 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 approximately 50% and purity ranged from 95%–99% depending on the antibody used to label the cells.

Natural cytotoxicity assay
This has been described in detail elsewhere (Brooks & Flannery, 1980) and has been shown to measure cytotoxicity due to natural killer cells when normal spleen cells are used as effectors with sarcoma Mc7 as target cells. Normal macrophages can also contribute to cytotoxicity but can be removed by treating the effector cells with carbonyl iron (Brooks, Flannery, Webb & Baldwin, 1980a). Briefly, 5×10^3 ^{51}Cr-labelled Mc7 cells were mixed with graded numbers of effector cells in a total volume of 300 μl MEM/10% FCS in the wells of microtest II plates (Sterilin, England). After a 6 hr incubation at 37° in 5% CO_2, 100 μl of supernatant was removed and counted on a gamma counter.

$$^{51}\text{Cr release } (\%) = \frac{100 \times \text{c.p.m. in supernatant}}{\text{total c.p.m. in tumour cells}},$$

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

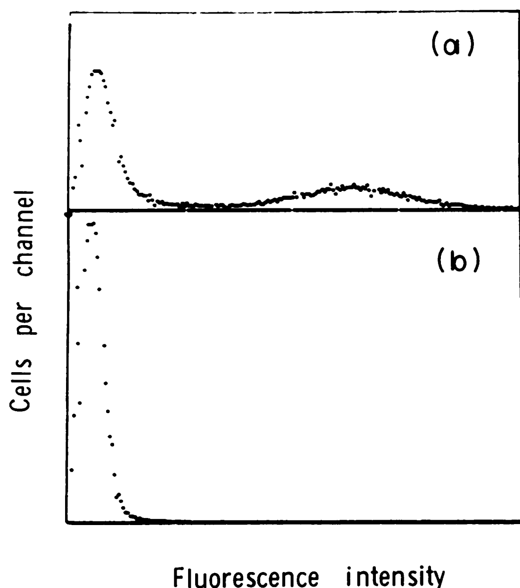

Figure 1. Fluorescence histograms obtained from the FACS using spleen lymphocytes labelled with W3/25 monoclonal antibody. Percentage staining before depletion of W3/25 + cells (a) was 40%, percentage staining after depletion (b), 0·8%

Analysis of results
For sarcoma Mc7, when percentage cytotoxicity (up to 25%) is plotted against effector cell concentrations, the slope of the regression line is directly proportional to the frequency of cytotoxic cells in the effector population (Brooks & Flannery, 1980). The relative cytotoxic activity (RCA) of two cell populations is, therefore, calculated as 100 S1/S2, where S1 is the slope of the regression line for the test cells and S2 the slope for control cells. The recovery of lytic units is then given by $y \times$ (RCA), where y is the fractional yield of cells in the test group compared with the control group. Statistical analysis was performed using Students' t test.

RESULTS

Table 1 shows the results of experiments where spleen cells are depleted of OX8 positive and W2/25 positive cells by rosette formation and resultant populations assayed for NK activity. Following depletion with OX8 antibody there was a significant reduction in the relative cytotoxic activity of a spleen cell population. In contrast, depletion with W3/25 antibody resulted in enrichment for cytotoxicity. Lytic unit analysis revealed no loss of cytotoxic activity in the W3/25 negative population but a 70%–80% loss of activity in

Table 1. Splenic NK activity after rosette depletion using OX8 and W3/25 monoclonal antibodies

Antibody	Cytotoxicity* Control¶	Cytotoxicity* Depleted	Relative cytotoxic† activity (RCA)	Yield‡	Lytic unit§ recovery
OX8	1·32±0·11	0·43±0·05**	32	82	26
	2·18±0·25	0·55±0·08**	25	74	19
	††3·61±0·37	1·65±0·17**	45	59	26
	1·32±0·11	3·05±0·36**	230	51	117
W3/25	3·51±0·33	5·6 ±0·54**	160	51	82
	3·61±0·37	6·29±0·93**	175	52	92

* Slopes ± SD of regression line for cytotoxicity.
† Ratio of the slopes of regression line for cytotoxicity.
‡ Expressed as a percentage of control cell yields.
§ Lytic unit recovery = RCA × yield.
¶ Control cells were unlabelled spleen cells rosetted in the same way as the spleen cells labelled with monoclonal antibody
** P = 0·05
†† Spleen cells pretreated with carbonyl iron

Table 2. NK activity of spleen cells after labelling with monoclonal antibodies and separation by fluorescence-activated cell sorter

Spleen§ cell fraction	Cytotoxic activity (slopes ± SD × 10^5)	RCA*	Yield†	Recovery of‡ lytic units
Unfractionated¶	2·70±0·12	—	—	—
OX8 positive	5·84±0·10**	217	26	57††
OX8 negative	0·57±0·10**	20·9	68	14
Unfractionated	2·03±0·30	—	—	—
W3/25 positive	0·02±0·06**	1·05	46	0·48
W3/25 negative	3·43±0·13**	169	54	90
Unfractionated	3·01±0·13	—	—	—
W3/13 positive	1·27±0·06**	42	70	30††
W3/13 negative	6·55±0·37**	217	26	57

* Ratio of the slopes of regression lines for cytotoxicity.
† Percentage of stained/non-stained cells.
‡ Product of the yield × RCA.
§ Spleens were pretreated with carbonyl iron to exclude any macrophages involvement before FACS separation.
¶ Control cells were subjected to treatment identical to that received by the fractionated population, i.e. labelled with antibody and run through the FACS without sorting. There was no significant difference between the cytotoxicity activity of these control cells and non-treated spleen cells.
** $P = 0{\cdot}05$
†† The data are representative results from three experiments in the case of OX8 and W3/13 antibodies. The mean recovery of lytic units in OX8 postive spleen cells was 66% and in OX8 negative spleen cells 10%. The mean recovery in W3/13 positive cells being 41% and in W3/13 negative cells being 50%.

Table 3. Splenic NK activity after rosette depletion of OX8 positive cells and FACS separation using W3/13 monoclonal antibody

Cell fraction‡	Cytotoxic Activity (slopes ± SD × 10^5)	RCA*	Cell yield	Recovery of† lytic units
Unfractionated	4·54±0·31	—	—	—
OX8 − ve	1·45±0·05	31§	71§	22§
OX8 − ve W3/13 − ve	1·73±0·19	119¶	39¶	46¶
OX − ve W3/13 + ve	1·87±0·15	129¶	52¶	67¶

* Ratio of slopes for cytotoxicity.
† Product of the yield × RCA.
‡ Spleen cells were pretreated with carbonyl iron to exclude macrophage involvement.
§ RCA, yield, lytic unit recovery relative to the unfractionated spleen cells.
¶ RCA, yield, lytic unit recovery relative to OX8 negative spleen cells.

the OX8 negative population. Carbonyl iron treatment had no significant effect on the residual cytotoxic activity of the OX8 negative populations indicating that it was not due to macrophages.

The data suggest that rat splenic NK cells are W3/25 negative but that the majority are OX8 positive. However, direct evidence that OX8 positive spleen cells had NK activity was not available until experiments were carried out using the fluorescence-activated cell sorter. Carbonyl iron treated spleen cells were sorted into OX8 positive and OX8 negative populations and the two groups assayed for cytotoxicity. The data (Table 2) show that OX8 positive spleen cells had enriched cytotoxic activity and OX8 negative spleen cells depleted cytotoxicity.

A similar experiment was carried out using W3/25 monoclonal antibody to effect a separation on the FACS. The data (Table 2) confirmed the rosetting data in that all the NK activity was recovered in the W3/25 negative spleen cell population and, as predicted, the W3/25 positive spleen cell populations showed no significant cytotoxicity.

In additional experiments, the NK activity of W3/13 positive and W3/13 negative spleen cell populations was examined. Separations were carried out using the FACS and not by rosette depletion because when using W3/13 monoclonal antibody, yields from the rosette depletions were consistently lower than 40% of theoretical, which invalidates lytic unit analysis. However, the data from the FACS separations indicated that W3/13 monoclonal antibody does label rat NK cells with 50% of NK activity due to W3/13 negative spleen cells and 40% due to W3/13 positive spleen cells (Table 2).

It is therefore clear that rat NK cells are heterogeneous with respect to their surface markers. The data show that the OX8 positive NK cell subpopulation contains both W3/13 positive and W3/13 negative

Table 4. Cytotoxic activity of BCG-activated NK cells separated on the basis of their expression of W3/25 and MRC OX8 defined antigens

Cell fraction	Separation technique	Cytotoxic activity (slopes ± SD × 10^5)	Lytic unit* recovery
Unfractionated	Rosetting	4·31±0·66	—
OX8 − ve	Rosetting	1·18±0·01†	19
W3/25 − ve	Rosetting	7·51±0·27†	98
Unfractionated	FACS	5·31±0·44	—
OX8 − ve	FACS	1·1 ±0·25†	14
OX8 + ve	FACS	14·3 ±0·37†	71

* Product of the fractional yield of cells and their relative cytotoxic activity to control cells.
† $P = <0.05$.

cells. It is not clear, however, whether the OX8 negative cell population shows a similar heterogeneity with respect to W3/13. To clarify this point, splenic NK cells were depleted of OX8 positive cells by the rosette-depletion technique and the resultant population sorted with W3/13 on the FACS. The data (Table 3) show that 22% of the NK cells were OX8 negative and of these approximately 60% were W3/13 positive and 40% W3/13 negative.

To demonstrate further that rat NK cells label with W3/13 and OX8 but not W3/25 monoclonal antibodies, the antigen expression of BCG-activated cells was analysed. The data (Table 4) show that BCG-activated NK cells have a similar antigen expression to resident splenic NK cells with respect to OX8 and W3/25 defined antigens (W3/25 −ve, 80% OX8 +ve). However, with respect to the W3/13 defined antigen, the analysis of the BCG-activated NK cells was technically difficult. This is because, unlike spleen cells, carbonyl iron treated BCG-induced peritoneal

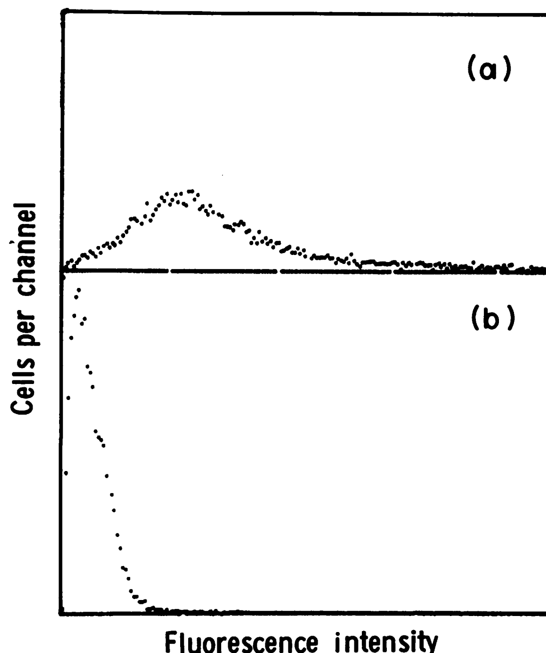

Figure 2. Fluorescence histograms obtained from the FACS using carbonyl iron treated BCG-induced peritoneal exudate cells (a) shows cells incubated first with W3/13 monoclonal antibody and then fluorescein-labelled rabbit anti-mouse immunoglobulin (b) shows control cells incubated with fluorescein-labelled rabbit anti-mouse immunoglobulin alone.

exudate cells show a continuous distribution of staining with W3/13 monoclonal antibody (Fig. 2) and there is no discrete negative population of cells. This indicates, but does not prove, that BCG induced NK cells are W3/13 positive.

DISCUSSION

The results establish that splenic NK cells express the T cell-associated antigens defined by OX8 and W3/13 monoclonal antibodies. BCG-induced NK cells also label with OX8, but their phenotype with respect to W3/13 is more difficult to establish. It seems probable that they are W3/13 positive, but it cannot be excluded that W3/13 negative cells contribute to the NK-cell cytotoxicity. These data are not evidence that NK cells share the T-cell differentiation pathway because neither OX8 or W3/13, nor indeed W3/25 antibodies are restricted entirely to cells of a T lineage. All three monoclonals label spleen cells in athymic rats in which there are marked T-cell deficiencies (Brooks *et al.*, 1980; Brideau *et al.*, 1980). Furthermore W3/25 has been shown to label macrophages (Brideau *et al.*, 1980) and W3/13 polymorph and granulocyte precursors in bone marrow (Williams *et al.*, 1977).

Regardless of the differentiation pathway of rat NK cells it is apparent from the present study that they are heterogeneous with respect to their surface markers. Thus there are both OX8 positive and OX8 negative NK-cell populations. Also, both the OX8 positive and OX8 negative NK cells are heterogeneous with respect to the W3/13 defined antigen. The finding that rat NK cells are heterogeneous is consistent with previous studies of murine and human NK cells (Koo, Jacobson, Hammerling & Hammerling, 1980; Zarling & Kung, 1980). Interestingly, in human systems it appears that the different NK-cell populations may recognize different antigenic specificities (Phillips, Ortaldo & Herberman, 1980). Whether this is also the situation with the rat NK cell remains to be investigated although it has been shown that natural immunity to both solid and lymphoid tumour target cells is mediated by cells with identical phenotypes as defined by W3/13, W3/25 and OX8 antibodies (D. A. Cantrell & C. G. Brooks unpublished data).

One of the major conclusions of this study, that rat NK cells can fall into the OX8 positive category of lymphocytes, has two important implications. Firstly, it presents the first evidence that OX8 monoclonal antibody labels functional subpopulations of lymphocytes

other than mature T cells. Secondly, it suggests that the hypothesis that OX8 positive cells in the rat are analogous to Ly23 positive cells in the mouse (Brideau *et al.*, 1980) is incorrect. Thus although in the rat, NK cells and cytotoxic T cells overlap and are both labelled with OX8 antibody, in murine systems there is no overlap and Ly23 is restricted entirely to the T cell-effector population (Herberman, Djeu, Kay, Ortaldo, Riccardi, Bonnard, Holden, Fagnani, Sanotini & Puccetti, 1979). It is clear, therefore, that in rat systems it is not possible to dissociate NK cells and cytotoxic T cells on the basis of the surface antigen markers so far defined. This has clear significance for the study of cytotoxic effector systems in the rat, especially with regard to tumour studies where often the object of investigation is to determine the relative contribution of NK cells and cytotoxic T cells to tumour cell lysis. It is obvious that in such systems, the use of OX8 or W3/13 as T cell markers is inappropriate.

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