

# Evidence by Reactivity with Hybridoma Antibodies for a Probable Myeloid Origin of Peripheral Blood Cells Active in Natural Cytotoxicity and Antibody-dependent Cell-mediated Cytotoxicity

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**ABSTRACT** Lymphocytes with Fc receptors (FcR) for IgG active in natural cytotoxicity and antibody-dependent cellular cytotoxicity were separated into sheep erythrocyte rosetting ( $E^+$ ) and nonrosetting ( $E^-$ ) fractions, and examined for reactivity with the OK panel of hybridoma-produced monoclonal antibodies. Few cells in either the  $E^+$  FcR $^+$  or the  $E^-$  FcR $^+$  fraction reacted with seven antibodies used to define T cells in various stages of differentiation (OKT3, OKT4, OKT5, OKT6, OKT8, OKT9, OKT10). Neither fraction expressed an Ia-like antigen (detected by OKI1), but both were highly reactive with OKM1, an antibody that reacts with monocytes and granulocytes. Incubation of these cytotoxic effector cells with OKM1 plus complement abolished all cytotoxic reactivity, but incubation with a pan-T cell antibody (OKT3) plus complement had no significant effect. These cells were not monocyte precursors, because they could not be induced in vitro to develop macrophage characteristics. The data indicate that most cytotoxic effector cells in natural cytotoxicity and antibody-dependent cellular cytotoxicity are not in the T cell lineage, but have a myeloid origin.

## INTRODUCTION

The precise lineage of blood lymphocytes that kill certain target cells in vitro without known prior sensitization to those targets (natural cytotoxicity, NC)<sup>1</sup> and of lymphocytes that kill immunoglobulin G (IgG)-sensitized target cells (antibody-dependent cellular

cytotoxicity, ADCC) is not well understood. Although it is agreed that lymphocytes with both NC and ADCC activities, when freshly isolated from blood, possess membrane Fc receptors (FcR) for IgG (1), further characterization of these cells has been controversial. Originally, it was reported that the C3 receptor-negative subset of non-T, non-B ("null") cells (2), which we have called L cells (3), were the cytotoxic effector cells. However, recent evidence has suggested that these cells may also have a T cell lineage. They have been shown to form rosettes with sheep erythrocytes (1, 4), and, in other laboratories, their cytolytic activity has been abrogated by incubation with heterologous anti-T cell antisera and complement (5). In this study, the relationship of cytotoxic effector cells to T cells was more precisely determined by isolating the FcR-bearing effector cells in both the erythrocyte rosetting  $E^+$  and the nonrosetting  $E^-$  fractions and examining them in parallel assays for NC and ADCC activities, as well as for reactivity with a panel of nine monoclonal antibodies that defined various stages in the developmental lineage of T cells (6-8).

## METHODS

*Peripheral blood lymphocytes.* Procedures have been described (1, 4) to isolate mononuclear leukocytes from peripheral blood, to remove monocytes, and to separate lymphocytes that formed rosettes with sheep erythrocytes ( $E^+$  cells from  $E^-$  cells). Greater than 95% of lymphocytes in  $E^+$  pellets formed rosettes, whereas only 4-6% of lymphocytes in the interface did so. Lymphocytes bearing high-avidity FcR for IgG in the  $E^+$  and  $E^-$  fractions were isolated by adherence to bovine serum albumin (BSA)-antiBSA immune complexes immobilized on plastic petri dishes, as previously described (9).  $E^+$  cells with low-avidity FcR for IgG, and also B cells, did not adhere to these immunobilized complexes, as has already been reported (10). Hence, FcR $^+$  cells, as described in this report, only refer to cells bearing high-avidity FcR for IgG. Greater than 90% of the

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<sup>1</sup>Abbreviations used in this paper: ADCC, antibody-dependent cellular cytotoxicity; BSA, bovine serum albumin; FcR, Fc receptors; L cells, C3 receptor-negative subset of non-T, non-B ("null") cells; NC, natural cytotoxicity.

starting number of lymphocytes were recovered after these adherence procedures. 5–8% of the total E<sup>+</sup> fraction were recovered in the FcR<sup>+</sup> subset, whereas 40–60% of the E<sup>-</sup> fraction were similarly recovered. Less than 2% of the E<sup>-</sup>FcR<sup>+</sup> cells expressed surface immunoglobulin, as was expected. Lymphocytes were stained with monoclonal antibodies while still bound to the immobilized complexes, or after they had detached spontaneously after overnight incubation on the complexes. Their reactivities with the antibodies were identical in both cases.

**Monoclonal antibodies.** The hybridoma-produced, monoclonal antibodies used in these studies were obtained from Doctors Patrick Kung and Gideon Goldstein, Ortho Pharmaceutical Corp., Raritan, N. J. The production, growth, and characterization of these antibodies have been the subjects of a series of recent reports (6, 7).

**Immunofluorescence procedures.**  $2 \times 10^5$  cells in 50  $\mu$ l, and 100  $\mu$ l of a 1:300 dilution of the monoclonal antibodies were mixed at 4 C for 30 min, washed 3 $\times$ , and developed with an fluorescein isothiocyanate labeled IgG fraction of goat-anti-mouse IgG (Meloy Laboratories Inc., Springfield, Va.). From 100 to 200 cells in each cell suspension were examined by fluorescence microscopy, and the percentages of reactive cells were determined. In three of these experiments, cells were simultaneously analyzed by flow cytometry, using a fluorescence-activated cell sorter (FACS IV, Becton, Dickinson, & Co. Rutherford, N. J.).

**Detection of monocyte-macrophagelike cells.** Cyto-centrifuge preparations of each lymphocyte suspension were stained for  $\alpha$ -naphthyl esterase, using  $\alpha$ -naphthyl acetate as the substrate. Usually <1–2% of the cells were stained in these preparations. In addition, L cells and E<sup>+</sup> cells bearing high-avidity FcR (Ty cells) were placed into culture for up to 9 d and examined daily for morphological changes, development of esterase reactivity, and capacity to ingest 0.81- $\mu$ m latex particles (Difco Laboratories, Detroit, Mich.). Less than 1% of the cultured cells were macrophages by any of these criteria.

**Cytotoxicity assays.** The 4 h <sup>51</sup>Cr release assay was performed exactly as described (1), using <sup>51</sup>Cr-labeled K562 cells for the NC assay, and antibody-sensitized Chang cells for the ADCC assay. Cytotoxicity was expressed as lytic units. One lytic unit represented the number of effector cells required to release 30% of the <sup>51</sup>Cr from the target cells, as previously described (1).

Complement-mediated lymphocytolysis was performed by incubating  $6-7 \times 10^6$  lymphocytes in 100  $\mu$ l of medium (RPMI 1640 containing 0.3% BSA and no HEPES buffer) with 200  $\mu$ l of either OKT3 antibody (final dilution 1:100), OKM1 antibody (final dilution 1:20), or medium alone. After 60 min at 4°C, treated cells were washed once, incubated for 120 min at 37°C with a 1:8 final dilution of rabbit complement (Pel-Freeze Biologicals, Inc., Rogers, Ark.). Less than 5% of the starting number of immunofluorescent cells remained after reaction with antibody and complement. When percent recovery of viable cells was determined, only from 30–40% of the cells treated with OKT3 and complement were recovered, whereas >80% of the cells treated with OKM1 and complement remained viable.

## RESULTS

Consistent with earlier reports (1, 4), Table I shows that NC and ADCC activities were found in both the E<sup>+</sup> (Ty) and the E<sup>-</sup> (non-T) fractions of freshly isolated peripheral blood lymphocytes. When each fraction was adjusted to the same number of effector cells per tar-

TABLE I  
Cytotoxic Capacity of E<sup>+</sup> and E<sup>-</sup> Lymphocyte Preparations before and after Adsorption on Plastic-Immobilized Immune Complexes

Lymphocyte subset†	Cytotoxic activity Number of lytic units* per 10 <sup>7</sup> effector cells	
	NK‡	ADCC
Unfractionated lymphocytes	65	75
E <sup>+</sup> Lymphocytes	46	54
E <sup>+</sup> FcR <sup>+</sup> subset	60	18
E <sup>+</sup> FcR <sup>-</sup> subset	2	<1
E <sup>-</sup> Lymphocytes	67	83
E <sup>-</sup> FcR <sup>+</sup> subset	80	24
E <sup>-</sup> FcR <sup>-</sup> subset	<1	<1

\* 1 lytic unit represents that number of lymphocytes required to cause the release of 30% of the <sup>51</sup>Cr from labeled targets in a 4-h assay.

† Lymphocyte suspensions had been depleted of monocytes by adherence on plastic surfaces, then separated into E<sup>+</sup> and E<sup>-</sup> subsets by standard rosette separation with sheep erythrocytes, and finally separated into FcR<sup>+</sup> and FcR<sup>-</sup> subsets by adsorption to plastic surfaces coated with BSA-antiBSA complexes. Effector cell suspensions contained <1% monocytes by esterase staining and latex-particle ingestion.

‡ NK, natural killer assay against K562 target cells; ADCC, antibody-dependent cell-mediated cytotoxicity against antibody-sensitized Chang cells.

get cell, the E<sup>-</sup> cells had more lytic activity per cell than did the E<sup>+</sup> cells. Table I, representative of seven similar experiments, further demonstrates that cytotoxic activity resided only in those subsets which had high-avidity FcR capable of binding to immobilized immune complexes. The nonbinding cells, which lacked FcR, or only expressed low-avidity FcR, expressed little or no cytotoxic activity. Thus, by collecting the FcR<sup>+</sup> cells which had bound to the immobilized complexes, we were able to examine with a panel of monoclonal antibodies those lymphocytes which had been shown to contain the cytotoxic effector cells.

In 11 experiments, 82% (range 72–90) of the total E<sup>+</sup> cell populations reacted with OKT3, a monoclonal antibody described as reacting with all peripheral blood T cells (6, 7). However, only 6% (range 0 to 21) of the E<sup>+</sup> FcR<sup>+</sup> subset of cytotoxic effector cells were stained by this monoclonal antibody in 15 separate experiments, including 3 done with FACS analysis (Table II). Less than 5% were stained by monoclonal antibodies that recognized T helper-inducer (OKT4) or cytotoxic-suppressor (OKT5, OKT8) cells. After removal of FcR<sup>+</sup> cells from the total E<sup>+</sup> fraction, however, the number of OKT3<sup>+</sup> cells in the remaining

FcR<sup>-</sup> cells increased from 82 to 95% ( $P = 0.01$ , Wilcoxon rank-sum test).

As expected, <2% of the E<sup>-</sup> FcR<sup>+</sup> cells reacted with the anti-T cell monoclonal antibodies. Thus, both E<sup>+</sup> and E<sup>-</sup> cytotoxic effector cells were similar in their very weak or negative reactivity with the panel of monoclonal antibodies used to define various T cell subsets.

There were other similarities between the E<sup>+</sup> FcR<sup>+</sup> and the E<sup>-</sup> FcR<sup>+</sup> subsets. Less than 10% of both reacted with OKT10, which detects an antigen found on T lineage cells from prothymocytes to thymocytes (6, 7), while <5% of either subset expressed an Ia-like antigen detected by OKI1, an antibody that reacts with B cells and monocytes (6). Both E<sup>+</sup> and E<sup>-</sup> cytotoxic effector cells were enriched in cells reacting with OKM1, an antibody that reacts with both monocytes and granulocytes (8). Since the total E<sup>+</sup> fraction contained only 8% OKM1<sup>+</sup> cells, almost all of these cells were therefore concentrated in the FcR<sup>+</sup> subset. Twice as many E<sup>+</sup> FcR<sup>+</sup> cells reacted with OKM1 as did E<sup>-</sup> FcR<sup>+</sup> cells ( $P = 0.01$ , Wilcoxon rank-sum test). As shown in Table III, incubation of effector cell suspensions with OKM1

(1:20 final dilution) and complement significantly inhibited NC activity against K562 cells, whereas incubation with OKM1 alone, OKT3 alone, or OKT3 plus complement was ineffective. When antibody-coated Chang cells were used as targets in ADCC, results identical to those obtained in the NC assays were obtained (data not shown).

Because reactivity with OKM1 raised the possibility of monocyte lineage for the effector cells, E<sup>+</sup> FcR<sup>+</sup> and E<sup>-</sup> FcR<sup>+</sup> cells were cultured in vitro for up to 9 d and examined at 3-d intervals for the ability to ingest latex particles, and for diffuse staining by  $\alpha$ -naphthyl esterase. Less than 1% of these cells possessed either of these characteristics.

## DISCUSSION

The results of the present study indicate that most FcR<sup>+</sup> cytotoxic effector cells in NC and ADCC, while present in both the E<sup>+</sup> and the E<sup>-</sup> fractions of peripheral blood, are not in the T cell lineage, as defined by the OK panel of hybridoma-produced monoclonal antibodies. These effector cells do, however, express an antigen (identified by the OKM1 antibody) that is not present on the FcR<sup>-</sup> fractions of blood lymphocytes that lack cytolytic activity.

As reported elsewhere (6–8), various T cell subsets have been defined by correlating functional activities of isolated cells with the surface antigens recognized on them by the monoclonal antibodies. By such correlations, then, the OKT3 antibodies have been shown to recognize almost all peripheral blood T cells; OKT4 antibodies bind to T helper cells and inducers of antigen-specific cytotoxic cells; OKT5 and OKT8 define

TABLE II  
Profile of Surface Markers Detected by Monoclonal Antibodies on Membranes of NK and ADCC Effector Cells

Monoclonal antibody† (and cell subset stained)	Percent of cells reactive with monoclonal antibody*			
	Enriched for cytotoxic effector cells		Depleted of cytotoxic effector cells	
	E <sup>+</sup> FcR <sup>+</sup>	E <sup>-</sup> FcR <sup>+</sup>	E <sup>+</sup> FcR <sup>-</sup>	E <sup>-</sup> FcR <sup>-</sup>
OKT3 (total T)	6±6§	2±2	95±3	8±5
OKT4 (helper/inducer)	5±5	1±1	64±8	4±3
OKT5 (cytotoxic/suppressor)	4±2	1±1	28±6	4±3
OKT8 (cytotoxic/suppressor)	5±4	2±2	30±7	5±4
OKT10 (thymocyte)	5±4	8±5	3±3	7±6
OKM1 (monocyte-myeloid)	75±15	47±5	2±1	8±6
OKI1 (Ia-like)	2±2	9±9	2±1	60±15

\* Reactivity with a murine monoclonal antibody was detected by indirect immunofluorescence with an FITC-labeled IgG fraction of goat anti-mouse IgG (see Methods). At least 200 stained cells in each cell preparation were scored by fluorescence microscopy.

† OKT6 (6, 7) and OKT9 (6, 7) were also tested, but were always <1%+ on the normal donors.

§ Mean±SD of 15 experiments using OKT3; 7 experiments using the other monoclonals. In three experiments with OKT3, both visual fluorescence microscopy and FACS analysis were performed simultaneously and the results were identical.

TABLE III  
Effect of Treatment with Monoclonal Antibodies and Complement on Natural Cytotoxicity of Peripheral Blood Lymphocytes

Effector cell treatment*	Number of lytic units per 10 <sup>7</sup> effector cells		
	Mean	Range	P value†
Control (untreated)	73.4	(43–125)	—
OKT3 only	97.5	(51–143)	0.48
OKM1 only	64.2	(43–125)	0.75
C only	55.4	(32–83)	0.36
OKT3 + C	70.0	(42–129)	0.90
OKM1 + C	11.0	(<1–24)	0.03

\* Effector cells were incubated with medium only (control), antibody alone, complement (C) alone, or antibody followed by complement, as indicated. Cell numbers were not re-adjusted before use in the assays for NC and ADCC.

† Test samples were compared with control samples to obtain the P values, as determined by Student's *t* test, using 4 d.f. (five experiments).

suppressor/cytotoxic T cells; and OKT6 and OKT9 recognize separate differentiation antigens on immature thymocytes (6, 7). In addition, OKI1 antibodies bind cells expressing Ia-like antigens or DRw determinants; OKM1 antibodies recognize an antigen expressed on certain cells with monocytic or granulocytic origin (8). By reactivity with these antibodies, then, our experiments have revealed a common phenotype of surface markers on both E<sup>+</sup> and E<sup>-</sup> cytotoxic effector cells. Previously described as being FcR<sup>+</sup> and C3R<sup>-</sup> (1, 2, 4), we now report that they can be identified by their expression of the OKM1 marker; simultaneously, most effectors seem to lack all T cell differentiation antigens (OKT3, OKT4, OKT5, OKT6, OKT8, OKT9, OKT10), as well as Ia-like markers present on B cells and monocytes.

These studies suggest that because T $\gamma$  and L cells are so very similar in morphology, function, and phenotype, they may derive from the same hematopoietic lineage. Further, it seems clear that not all E<sup>+</sup> FcR<sup>+</sup> cells can be classified as so-called T $\gamma$  cells, as shown by failure of many of these cells to react with any of the anti-T cell antibodies. Unlike Reinherz and coworkers who reported that 5–50% of their E<sup>+</sup> FcR<sup>+</sup> cells were T cells as determined by reactivity with OKT3 (11), we found that, with one exception, <16% of E<sup>+</sup> FcR<sup>+</sup> cells reacted with OKT3. These differences may be explained by the different procedures used to isolate E<sup>+</sup> FcR<sup>+</sup> cells. It should be noted that we isolated these cells by adherence to immobilized immune complexes rather than by positive selection of bovine erythrocyte-IgG rosettes, as was done by Reinherz et al. (11). Because adherence procedures only select for cells with high-avidity FcR (9, 10), we found that our E<sup>+</sup> FcR<sup>+</sup> cells were quite homogeneous in their reactivity with OKT3, in contrast to the heterogeneous reactivity reported by Reinherz. Furthermore, we found that only 75–90% of the total E<sup>+</sup> fraction were reactive with OKT3, whereas Reinherz and Schlossman (6) reported this value to be 100%. We found that only when FcR<sup>+</sup> cells were removed by adherence to immobilized complexes did >95% of the remaining E<sup>+</sup> FcR<sup>-</sup> cells react with OKT3. This was to be expected, since the total E<sup>+</sup> fraction contained from 5 to 15% FcR<sup>+</sup> cells, which are mostly OKT3 negative.

Although only 47% of the E<sup>-</sup> FcR<sup>+</sup> subset reacted with OKM1, our findings that 75% of the E<sup>+</sup> FcR<sup>+</sup> cells reacted with OKM1 agree with those of Reinherz et al. (11). Furthermore, our data indicate that most, if not all, cytotoxic effector cells reside in these OKM1<sup>+</sup> subsets, since treatment of cells with OKM1 plus complement almost totally abrogated NC and ADCC. That this marker is not part of the cytolytic mechanism, however, was made clear by failure of OKM1 antibody alone to inhibit cytotoxic reactivity. With the data available to date, we cannot exclude that these OKM1<sup>+</sup>

cells may be heterogeneous in origin, containing a fraction of T cells or T cell precursors. If other investigators confirm the findings of Breard et al. (8) that OKM1 is unreactive with lymphocytes of T or B cell lineage, then the myeloid origin of effector cells would be confirmed.

Our observation that cytotoxic effector cells lacked Ia-like antigens, and failed to become phagocytic or esterase positive during 6–9 d of culture, is evidence against their being monocyte precursors. It is possible, however, that the culture conditions chosen may not have been optimal for differentiation of precursors into macrophages.

These studies support our earlier hypothesis that nonphagocytic mononuclear cells with high-avidity FcR described as L cells may not be typical lymphocytes, but may be part of a separate hematopoietic lineage. The observation that these cells possess features of both lymphocytes and monocytes suggests that they may occupy an intermediate position in ontogeny and phylogeny.

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