

PHENOTYPIC CHARACTERIZATION OF HUMAN  
CYTOLYTIC T LYMPHOCYTES  
IN MIXED LYMPHOCYTE CULTURE

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A number of cell surface markers are expressed on resting peripheral blood (PB) T cells (1). However, there are a number of surface markers that are expressed only in low proportions or not at all on resting T cells, but that are clearly expressed in varying proportions on activated T cells. Among these markers of activated T cells, the receptors for the Fc portion of IgG (Fc $\gamma$ R) are on small fractions of PB sheep erythrocyte (E)-rosetting cells, usually referred to as T $\gamma$  cells (2, 3). The proportions of Fc $\gamma$ R-positive cells increase markedly upon activation of the cell (4). In addition, Ia-like antigens are expressed on a small proportion of freshly isolated circulating T cells; these are included within the T $\gamma$  fraction (5). Again, upon activation of T cells, variable proportions will express Ia-like antigens (6-8). Recently, a monoclonal antibody (4F<sub>2</sub>) has been described which binds to a non-Ia, 120,000-mol wt cell surface antigen present on resting PB monocytes, but which is present in negligible amounts on resting PB lymphocytes (9, 10). However, the 4F<sub>2</sub> antigen is expressed on a large proportion of lymphocytes which have been activated by mitogen and/or alloantigen stimulation (9).

The precise identity of the mixed lymphocyte reaction (MLR)-activated specific cytotoxic T cell (CTL) in humans has not been characterized by currently available cell surface markers.

In this study we have analyzed MLR-activated T cells to determine the relationship between specific cytotoxicity and the phenotypic expression of these activation markers. The results indicate that CTL are found exclusively in a population of cells lacking detectable Fc $\gamma$ R (Fc $\gamma$ R<sup>-</sup>) but expressing 4F<sub>2</sub> antigen. By contrast, CTL were detectable in both the Ia-positive and Ia-negative population of activated T cells.

#### Materials and Methods

*Cell Separations.* Mononuclear cells were obtained from heparinized PB by standard Ficoll-Hypaque separation and adherent cells were removed from the mononuclear cell suspensions by adherence to plastic Petri dishes. E-rosetting lymphocytes were separated from nonrosetting cells by fractionation of rosetting cells over two sequential Ficoll-Hypaque centrifugations (11). T cells bearing receptors for IgM (T $\mu$ ) were isolated as previously described (12). Stimulator cells for the MLR were prepared by two sequential Ficoll-Hypaque fractionations of E-rosetting cells from mononuclear cells to obtain E-rosette-depleted cell suspensions. These cells were then irradiated with 5,000 rad.

**Cell Cultures.** MLR were set up by culturing purified T cells or T $\mu$  cells either in macrowells ( $2 \times 10^6$  cells) or in round-bottomed microwells ( $10^6$  cells) with equal numbers of irradiated allogeneic E-rosette-depleted cells in RPMI-1640 that contained 10% fetal calf serum. Controls included both responder and stimulator cells cultured alone. To evaluate the cell proliferation, tritiated thymidine ( $^3\text{H}$ ]TdR) was added ( $1 \mu\text{Ci/well}$ ) to triplicate microwell cultures 18 h before harvesting, and the  $^3\text{H}$ ]TdR uptake was determined in a scintillation counter. In parallel experiments cells were harvested after 7 d, passed through a Ficoll-Hypaque gradient to remove dead cells, and after washing twice, cells were counted and the percentages of E-rosetting cells determined. When the proportion of E-rosettes was  $>95\%$ , no additional purification was performed. When the proportion was  $<95\%$ , E-rosetting cells were further isolated by additional Ficoll-Hypaque density gradient centrifugations.

**Enumeration and Isolation of MLR-activated Cells Expressing Surface Fc $\gamma$ R, Ia, or 4F $_2$  Antigen.** Cells with surface Fc $\gamma$ R were isolated as previously described by a rosetting technique using ox erythrocytes sensitized with purified rabbit IgG antibodies (12). Rosetting cells (Fc $\gamma$ R $^+$ ) were isolated from nonrosetting cells (Fc $\gamma$ R $^-$ ) by pelleting them at least twice through Ficoll-Hypaque gradients. Ox erythrocytes were lysed with  $\text{NH}_4\text{Cl}$  buffer. The Fc $\gamma$ R $^-$  cells were sham-treated with  $\text{NH}_4\text{Cl}$ . Monoclonal antibodies 4F $_2$  and D1/12 were prepared and used as previously described (10, 13). For evaluation of the percentage of Ia $^+$  and 4F $_2^+$  cells and for their isolation, T cell suspensions were reacted with saturating amounts of monoclonal antibody. The D1/12 monoclonal anti-Ia antibody was kindly provided by Dr. R. Accolla and Dr. S. Carrel of the Ludwig Institute for Cancer Research, Epilanges sur Lausanne, Switzerland. A fluoresceinated goat anti-mouse antiserum was used as the second reagent in saturating concentrations for analysis on the fluorescence-activated cell sorter (FACS II; BD FACS Systems, Mountain View, Calif.). Background fluorescence was obtained by FACS analysis of the same cell suspensions incubated with a mouse gamma-2 globulin in place of D1/12 (Ia) or 4F $_2$  monoclonal antibodies. 4F $_2$  or D1/12-labeled cells were also enumerated and were exclusively isolated by means of a rosetting technique using ox erythrocytes coated with staphylococcal A protein (13). Both rosetting and nonrosetting cells were treated with  $\text{NH}_4\text{Cl}$  lysing buffer after isolation.

After activation in the MLR, the proportions of cells positive for Ia or 4F $_2$  antigen were similar in both FACS and rosetting analysis. The percentage of Ia $^+$  cells ranged from 30 to 50% of responder T cells; both small and large cells were Ia $^+$ . 4F $_2$  antigen-bearing lymphocytes were 40–60% of the responder T cells and were all large cells. Fc $\gamma$ R $^+$  cells ranged from 20 to 50% of the responder T cells of a 7-d MLR—both large and small cells expressed Fc $\gamma$ R.

**Cytotoxicity Assay.** E-rosetting T cells from a 7-d MLR or T cells fractionated according to surface Fc $\gamma$ R, Ia, or 4F $_2$  antigen were tested for their cytotoxic capacity against the relevant target cells, which had been stimulated for 3 d with phytohemagglutinin.  $5 \times 10^3$  target cells labeled with  $^{51}\text{Cr}$  were incubated with varying numbers of effector cells (from 1:1 to 100:1 ratios). The plates were centrifuged and then incubated for 4 h at  $37^\circ\text{C}$ . Cytolysis was assessed by counting the radioactivity of 0.1 ml supernate for 1 min in a scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The specific lysis was calculated according to the method of Cerottini et al. (14).

## Results and Discussion

Purified T lymphocytes were used as responder cells against allogeneic irradiated E-rosette-depleted cells. After a 7-d MLR, E-rosetting cells were isolated and further fractionated according to the presence of Fc $\gamma$ R, surface Ia, or 4F $_2$  antigen. Unfractionated responder T lymphocytes (total T cells) and the various cell fractions bearing or lacking the above-mentioned markers were analyzed for their cytotoxic capability against the relevant target cells. Fig. 1 shows a typical experiment. The presence of Ia-like antigens recognized by D1/12 monoclonal antibody did not discriminate cells manifesting specific cytotoxicity, because unfractionated responder cells, Ia $^+$ , and Ia $^-$  fractions killed the target with similar efficiency (Fig. 1). These experiments were performed four separate times with similar results. On the other hand, fractionation

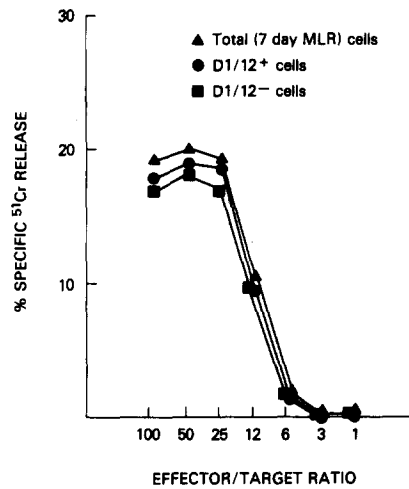


FIG. 1. Cytotoxic capacity of 7-d MLR T cells fractionated according to surface Ia antigens. The cytotoxicity induced by Ia<sup>+</sup> or Ia<sup>-</sup> effector T cells was similar to that of unfractionated T cells. Cells were fractionated by rosetting D1/12-coated responder cells with staphylococcal protein A-coated human erythrocytes. Similar results were obtained in four other experiments using either the rosetting technique or the fractionation by FACS II.

of responder T cells according to surface FcγR demonstrated that cytotoxic cells were present exclusively among the FcγR<sup>-</sup> fraction (Fig. 2). These experiments were performed 10 separate times with similar results. By contrast, cytotoxic T cells were restricted to the population bearing 4F<sub>2</sub> antigen (Fig. 3) as determined by five separate experiments. It is noteworthy that similar results were obtained in experiments in which purified Tμ cells were used as responders (data not shown). Thus, responder T cells that express none of these activation markers at the initiation of the MLR (purified Tμ cells) are equally capable of ultimately expressing them.

These results have clearly demonstrated that the MLR-generated CTL are contained in the 4F<sub>2</sub><sup>+</sup> and FcγR<sup>-</sup> cell fractions. Therefore, it is possible, after MLR activation, to selectively enrich or deplete specific CTL on the basis of the presence or absence of different activation markers.

Because CTL are restricted to the cells bearing the 4F<sub>2</sub> antigen, but lacking detectable FcγR, an obvious question is whether all the 4F<sub>2</sub><sup>+</sup> cells are FcγR<sup>-</sup> and, conversely, whether all of the FcγR<sup>-</sup> cells express 4F<sub>2</sub> antigen. Preliminary experiments have shown that this is not the case and that 4F<sub>2</sub><sup>+</sup> cells include variable proportions of FcγR<sup>+</sup> cells (up to 30%), whereas FcγR<sup>-</sup> cells can comprise up to 50% of 4F<sub>2</sub><sup>-</sup> cells. Thus, the combined use of FcγR and 4F<sub>2</sub> antibody allows one to further restrict the cytotoxic cells to the 4F<sub>2</sub><sup>+</sup>, FcγR<sup>-</sup> fraction, which usually accounts for 30–40% of the responder T cells.

As the fraction of cells that expresses 4F<sub>2</sub> antigen includes all the specific CTL, the question arises whether this surface structure is directly involved in the cytotoxic capacity of the cells. In this regard, addition of high concentration of 4F<sub>2</sub> monoclonal antibody to the cytolytic assay had no effect on the target cell killing. In addition, it is possible that the positive selection of FcγR<sup>+</sup> cells generated in MLR by rosetting techniques could affect the cytotoxic capacity of the cells themselves, because it is known that interaction of PB Tγ cells with IgG immune complexes induces loss of

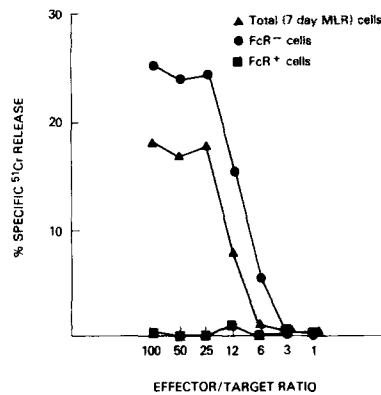


FIG. 2. Cytotoxic activity of 7-d MLR responder T cells fractionated according to surface Fc $\gamma$ R. Cells cytotoxic against the specific target were present exclusively in the Fc $\gamma$ R<sup>-</sup> fraction of activated T cells.

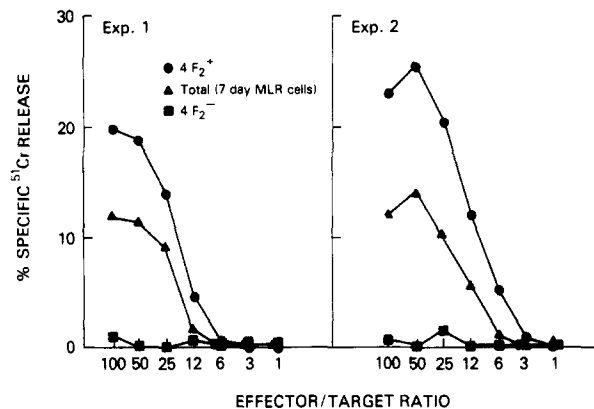


FIG. 3. Cytotoxic activity of 7-d MLR T cells fractionated according to the expression of 4F<sub>2</sub> antigen. Cells cytotoxic against the specific target were present exclusively in the 4F<sub>2</sub><sup>+</sup> fraction.

detectable cell surface Fc $\gamma$ R (15), and that modulation of Fc $\gamma$ R interferes with certain functional activities of the cells, such as responses to allogeneic cells or to T cell mitogens (16, 17). However, Fc $\gamma$ R<sup>+</sup> cells generated in MLR do not lose their Fc $\gamma$ R upon interaction with IgG immune complexes, and most importantly, they are extremely effective in antibody-dependent cellular cytotoxicity and natural killer cell assays (Mingari et al. Manuscript in preparation.). In addition, the Fc $\gamma$ R<sup>-</sup> cell fraction was always more active than the unfractionated population, further suggesting that specific CTL are indeed poorly represented in the Fc $\gamma$ R<sup>+</sup> fraction.

The precise nature and function of the Fc $\gamma$ R<sup>+</sup> cells that were generated in the MLR are still uncertain. There is little doubt about the T cell nature of these cells because they express various T cell markers (Mingari et al. Manuscript in preparation.). In addition, these MLR-generated T $\gamma$  cells are detectable in high proportions even when the responder cells are T $\gamma$  depleted before the inception of the MLR as was done in the experiments employing purified T $\mu$  cells as responder cells. This would rule out the possibility of an expansion of cytotoxic cells originally present in the T $\gamma$  fraction as a consequence of the release of nonspecific growth factors during the course of the MLR.

### Summary

Mixed lymphocyte reaction (MLR)-activated T cells were analyzed according to the expression of various cell surface markers by the specific cytotoxic T lymphocytes (CTL) generated in the MLR. CTL were found exclusively in a population of MLR-activated T cells that lacked detectable Fc $\gamma$ R but that expressed a surface antigen recognized by the 4F<sub>2</sub> monoclonal antibody. In contrast, CTL were found in both the Ia-positive and Ia-negative cells after MLR activation. Thus, the specific CTL generated in the allogeneic MLR can be identified and isolated by virtue of the expression of a particular cell surface marker.

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