

**SURFACE MARKERS OF CLONED HUMAN T CELLS  
WITH VARIOUS CYTOLYTIC ACTIVITIES**

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Several surface membrane markers have recently been described that define T cell subpopulations in man. For example, receptors for immunoglobulin (Ig) isotypes (1) and membrane structures defined by xenoantisera (2) and, more recently, monoclonal antibodies (3), have been used to identify various subsets in peripheral T cell populations. Other markers, such as Ia antigens (4) and a group of surface antigens recognized by monoclonal antibodies (5, 6), appear to be expressed on T cells primarily upon cell activation. Functional studies of T cell subsets have favored the idea that there is a link between the expression of given markers and T cell function (1-3). However, a precise correlation between surface markers and cell function may be difficult if only a small percentage of the cells in a given subset is involved in the functional activity measured. It thus appears difficult to deduce the function of individual cells from their surface markers.

In an attempt to correlate more precisely the surface phenotype of human T cells with a given functional activity, we have analyzed 14 cloned T cells with various cytotoxic activities for a battery of surface markers, namely receptors for sheep erythrocytes (E rosettes), receptors for the Fc portion of IgG or IgM (Fc $\gamma$ R and Fc $\mu$ R), and a group of antigens recognized by monoclonal antibodies, including Ia, 4F2 (6), OKT8, and OKT4 (3).

**Materials and Methods**

*Mixed Lymphocyte Cultures.* Lymphocytes were isolated from peripheral blood lymphocytes (PBL) or spleen as previously described (1). Cells were suspended in RPMI 1640 containing 10% heat-inactivated human AB serum. Spleen cell suspensions were frozen and subsequently thawed as needed. Primary mixed lymphocyte cultures (MLC) were set up using PBL as responder cells and irradiated allogeneic spleen cells as stimulator cells, as previously described (7). For the preparation of secondary MLC, cells from day 7 (primary) MLC were centrifuged on a Ficoll-Hypaque gradient to remove dead cells, and then mixed at a 1:10 ratio with irradiated stimulator spleen cells either in macrowells ( $4 \times 10^6$  total cells per well) or in tissue flasks ( $15 \times 10^6$  total cells per flask). After 4-5 d of culture, secondary MLC cells were harvested, passed through Ficoll-Hypaque gradients, and washed. The majority of the recovered cells were large cells (>70%) and formed rosettes with sheep erythrocytes (SRBC) (>85%).

*Cloning of MLC Cells.* Secondary MLC cells were submitted to vigorous vortex mixing (to dissociate possible cell clumps) and then seeded at 0.3 cells per well in round-bottomed microwells containing  $10^5$  irradiated stimulator spleen cells. The culture medium was RPMI 1640 containing 10% human AB serum and 50% supernate from phytohemagglutinin (PHA)-stimulated human spleen cell cultures (as a source of T cell growth factor [TCGF]) (8).

*Screening of Cytolytic Activities.* A preliminary analysis of the cytolytic activities of the clones

was performed as follows. Cells of "positive" wells were first resuspended with a pasteur pipette. Several 20- $\mu$ l aliquots of the cell suspensions were then transferred into V-bottomed wells of microtiter trays to which  $5 \times 10^3$   $^{51}\text{Cr}$ -labeled target cells were added (the final volume per well was 200  $\mu$ l). The target cells used were (a) PHA-induced blast cells derived from the stimulator cell population, (b) K562 human target cells, and (c) antibody-coated L1210 mouse cell line. The plates were centrifuged at 100 *g* for 5 min and then incubated for 4 h at 37°C. After centrifugation of the plates at 200 *g* for 5 min, cytotoxicity was assessed by counting the radioactivity of 0.1 ml supernate for 1 min in a scintillation counter. The specific lysis was calculated according to Cerottini et al. (9).

*Expansion of Clones.* Cells of selected clones were added to macrowells together with irradiated spleen cells ( $2 \times 10^6$ ) of the same donor as those used as stimulator cells in RPMI 1640 containing 10% human AB serum and 50% TCGF. Under these conditions, the majority of microcultures showed strong proliferation, generating a number of cells suitable for further analysis (average  $2 \times 10^6$  cells) in 7–12 d. Cells derived from the same clones were pooled, passed over Ficoll-Hypaque gradients, counted, and analyzed for cytolytic activities against the three types of target cells used in the initial selection and for surface markers.

*Analysis of Surface Receptors for SRBC and for the Fc Fragments of IgG or IgM (Fc $\gamma$ R and Fc $\mu$ R).* The techniques have been extensively described in a previous report (1). Neuraminidase-treated SRBC were used, and the proportion of rosetting cells was evaluated by scoring at least 400 cells. Fc $\gamma$ R- or Fc $\mu$ R-bearing cells were detected by rosetting assays using ox erythrocytes coated with purified IgG or IgM fractions of rabbit anti-ox erythrocyte antisera.

*Analysis of Surface Antigens by Monoclonal Antibodies.* The reactivity of cloned cell suspensions or normal PBL with PTF (anti-Ia) (a generous gift of Dr. G. Damiani), 4F2 (6), OKT8, and OKT4 (3), monoclonal antibodies was determined by indirect immunofluorescent staining using fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Flow cytometry of stained cells was performed on a fluorescence-activated cell sorter (B D FACS Systems, Mountain View, Calif.) as described previously (6).

*Subclones.* Subclones were obtained by seeding cells from two selected cytotoxic clones at 0.3 cells per well in microwells under the same culture conditions used for cloning. The cytolytic activities of eight expanded subclones derived from each clone were evaluated on the three types of target cells mentioned above.

## Results and Discussion

Peripheral blood T cells that had been stimulated twice in MLC were cultured under limiting conditions (0.3 cells per microwell) with irradiated allogeneic cells in the presence of a suitable source of TCGF (8). As assessed microscopically after 10–15 d, 7–22% of the wells contained proliferating cells. Each of such positive wells was tested for lytic activity on three different  $^{51}\text{Cr}$ -labeled target cell types, namely PHA-induced blast cells bearing the stimulating alloantigens (cytotoxic T lymphocytes [CTL] activity), L1210 murine lymphoma cells sensitized with rabbit antibody (antibody-dependent cell-mediated cytotoxicity [ADCC] activity), and K562 human cell line (natural killer [NK] activity). Cells from microcultures with lytic activity restricted to only one of the target cell types used were expanded for further analysis. The vast majority of the expanded clones were found to have maintained their original cytolytic pattern. The lytic activity of 14 such clones is shown in Table I. Four expanded clones that were devoid of cytolytic activity are included for comparison. It can be seen that 8, 4, and 2 of the 14 cytolytic clones tested displayed CTL, NK, or ADCC activity, respectively.

Two clones selected for their high CTL activity were subcloned by limiting dilution. From each parental clone, eight subclones were tested for lytic activity against the target cell types used in this study. All subclones exhibited a high lytic activity against the corresponding blast target cells without detectable NK or ADCC activity (data

TABLE I  
Cytolytic Activity and Surface Markers of Human T Cell Clones

Clone number	Cytolytic activity			Receptors			Antigens			
	CTL	NK	ADCC	SRBC	Fc $\gamma$ R	Fc $\mu$ R	Ia	4F2	OKT8	OKT4
1	2*	<u>86</u> ‡	3	<u>89</u> §	1	0	+	+	+	-
2	7	<u>46</u>	0	78	0	8	+	+	-	-
3	1	<u>75</u>	0	75	0	10	+	+	+	-
4	0	<u>80</u>	0	84	1	3	+	+	+	-
5	0	<u>0</u>	35	80	42	1	+	+	-	+
6	0	<u>0</u>	<u>41</u>	81	29	0	+	+	-	+
7	<u>39</u>	0	<u>0</u>	93	2	1	+	+	-	+
8	<u>82</u>	9	6	92	4	3	+	+	+	-
9	<u>88</u>	2	0	94	0	1	+	ND¶	-	ND
10	<u>51</u>	12	0	74	3	10	+	+	-	+
11	<u>35</u>	2	0	83	0	4	+	+	-	-
12	<u>27</u>	2	0	73	0	8	+	+	-	-
13	<u>26</u>	2	0	75	0	8	+	+	-	-
14	<u>60</u>	3	7	94	0	1	+	+	-	+
15	<u>0</u>	0	0	93	1	4	+	+	-	-
16	0	0	0	77	0	50	+	+	-	+
17	0	0	0	81	1	26	+	+	-	+
18	0	2	0	89	2	7	+	+	-	-

\* Percent of specific lysis at 30:1 lymphocyte per target ratio.

‡ Underlined numbers indicate clones with a given cytolytic activity.

§ Percent of rosette-forming cells.

|| Presence or absence of surface antigens was analyzed by fluorescence-activated cell sorter.

Virtually 100% of the cells of each clone were either positive or negative for a single antigen.

¶ ND, not determined.

not shown). Although these results are in support of clonality of the original isolates, they also demonstrate that the cytolytic pattern of the clones tested was stable under the conditions used for their expansion.

As shown in Table I, all clones were E rosette positive, with 73–94% of the cells in individual clones forming rosettes. The fact that a small percentage of these cells did not form E rosettes is probably due to limitations of the technique used. Alternatively, the possibility exists that cells at different stages of the cell cycle or with different metabolic activity vary in their expression of receptors for SRBC. In support of this contention is the finding that five individual subclones derived from the E rosette-negative fraction of a putative clonal isolate were mostly E rosette positive (unpublished observation).

In 12 cytolytic clones, Fc $\gamma$ R were virtually absent, whereas 30–40% of the cells in the two clones with ADCC activity were Fc $\gamma$ R positive. It is of interest that these two clones had no lytic activity against K562 target cells. Conversely, the four clones reactive against K562 target cells were Fc $\gamma$ R negative and devoid of K cell activity. It thus appears that ADCC and NK cell activities, although they are mediated by the same (Fc $\gamma$ R-positive) subset in PBL populations, can be dissociated in clones derived from alloreactive MLC populations. Flow cytometric analysis showed that all (cytolytic and noncytolytic) clones expressed relatively large amounts of Ia antigens. Virtually 100% of the cells in individual clones were Ia positive (Fig. 1). Similar results were obtained when the clones were analyzed for expression of 4F2 antigen. In contrast, only four of the cytolytic clones were OKT8<sup>+</sup>. Among these clones, three were cytolytic against K562 target cells, whereas one had CTL activity. Of the seven OKT8<sup>-</sup> CTL clones, three were OKT4<sup>+</sup>. The latter antigen was also expressed in the two clones active in ADCC and two of four noncytolytic clones. Flow cytometric

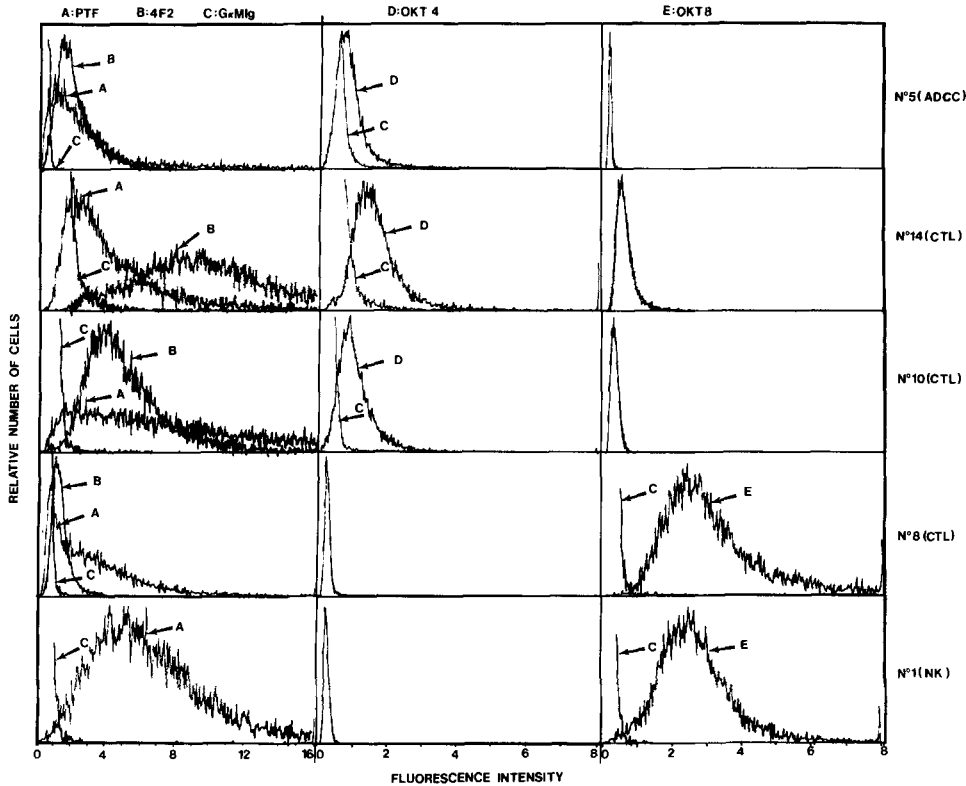


FIG. 1. Cell surface antigens of representative human T cell clones. Viable cells from clones with various cytolytic activities were collected after removal of dead cells by Ficoll-Hypaque gradients. These cells were stained with monoclonal PTF (anti-Ia) (A), 4F2 (B), OKT4 (D), and OKT8 (E) antibodies. Fluorescein-coupled goat anti-mouse immunoglobulin was used as a second reagent. The fluorescence of  $10^4$  viable cells was quantitated by using a fluorescence-activated cell sorter. Fluorescence distributions in arbitrary units are in each case compared with cells stained with the fluoresceinated goat anti-mouse immunoglobulins alone (C).

analysis of individual CTL clones indicated that virtually all cells were positive or negative for either OKT8 or OKT4 antigens (Fig. 1).

Previous studies at the population level have provided conflicting results as regards Ia expression in human CTL. Whereas Reinherz et al. (10) reported that CTL activity displayed by *in vitro* alloactivated T cell populations was restricted to Ia-negative lymphocytes, earlier work from our laboratory (6) indicated that Ia<sup>+</sup> and Ia<sup>-</sup> MLC subpopulations exhibited similar levels of specific cytolytic activity. Although it cannot be ruled out that the procedure used in the present study (including the use of TCGF) for the derivation of expanded clones may favor the proliferation of Ia-positive cells, it is more likely that these conflicting results can be explained by differences in sensitivity of the methods used for the detection and isolation of Ia<sup>+</sup> cells.

Previous studies have shown that CTL activity of MLC populations was restricted to an FcγR-negative subpopulation expressing 4F2 antigen (6). The present study confirms at the clonal level that CTL generated in MLC are 4F2<sup>+</sup>. It is evident,

however, that this antigen cannot be used as a specific marker for CTL because it is also expressed in noncytolytic clones (Table I). Moreover, these results are in agreement with our previous studies showing that CTL activity in MLC populations is confined to the Fc $\gamma$ R-negative fraction of the 4F2<sup>+</sup> subset (6).

Using monoclonal antibodies of the OKT series, Reinherz and Schlossman (3) reported that CTL generated in MLC expressed the OKT8<sup>+</sup>, OKT4<sup>-</sup> phenotype. Such a phenotype was observed in only one of the eight CTL clones analyzed in the present study. Moreover, three of the OKT8<sup>-</sup> CTL clones were found to express the OKT4 antigen. Although the number of CTL clones examined was limited, these results clearly indicate that caution should be exercised in assigning a given function to lymphocytes bearing either the OKT4 or the OKT8 antigen. Along this line, it is of interest that three of four clones reacting against K562 target cells were OKT8<sup>+</sup> and OKT4<sup>-</sup>, whereas the two clones with ADCC activity expressed the opposite phenotype. To further analyze the relationship between these markers and functional activity, we are currently investigating the cytolytic and/or helper activities of expanded clones derived from individual MLC cells positively and negatively selected (by cell sorting) on the basis of fluorescence with OKT4 or OKT8 antibodies.

### Summary

Human T cells stimulated in secondary allogeneic mixed lymphocyte culture (MLC) were cloned under limiting conditions in microculture systems using T cell growth factor and irradiated allogeneic cells. Clones with lytic activity against either phytohemagglutinin-induced blast cells bearing the stimulating alloantigen(s) (cytotoxic T lymphocyte [CTL] activity), L1210 mouse lymphoma cells coated with rabbit antibody (antibody-dependent cell-mediated cytotoxicity [ADCC]), or K562 human target cells were selected, expanded, and then analyzed for different surface markers, including rosette formation with sheep erythrocytes (E rosettes), receptors for the Fc portion of IgG or IgM (Fc $\gamma$ R and Fc $\mu$ R), and a group of antigens recognized by monoclonal antibodies including Ia, 4F2, OKT8, and OKT4. All the cytotoxic cells were E rosette<sup>+</sup>, Ia<sup>+</sup>, and 4F2<sup>+</sup>. Expression of Fc $\gamma$ R was restricted to the clones active in ADCC. CTL clones were either OKT8<sup>+</sup> or OKT8<sup>-</sup>. Furthermore, three of the OKT8<sup>-</sup> CTL clones were OKT4<sup>+</sup>. In addition, some cytolytic clones devoid of specific CTL activity were OKT8<sup>+</sup>. It thus appears that the claim that human CTL are OKT8<sup>+</sup>, OKT4<sup>-</sup>, and Ia<sup>-</sup> is not supported by the analysis of their phenotype at the clonal level.

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