THE Fc RECEPTOR ON THYMUS-DERIVED LYMPHOCYTES

III. Mixed Lymphocyte Reactivity and Cell-Mediated Lympholytic Activity of Fc⁻ and Fc⁺ T Lymphocytes*·‡

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Several studies have suggested that a high degree of functional heterogeneity exists within the thymus-derived (T) lymphocyte class. T lymphocytes have been shown to initiate graft-vs.-host reactions (1), generate proliferative and cytotoxic responses to alloantigens (2–5), and to exert amplifier, helper, and suppressor effects on both antibody production (5–7) and cellular immunity (3, 4, 8–11). It is not clear, however, whether this functional heterogeneity reflects the ability of T cells to differentiate into distinct, functionally committed lineages either before or as a result of antigen presentation, or whether it reflects the functional differences arising from antigen presentation on one lineage of T cells at different stages of maturation.

A large number of cytotoxic and fluorescent probes reactive with T-cell membrane components have been prepared in attempts to delineate T-cell subpopulations and their maturational stages (1, 5, 12–16). We have used, as such a probe, fluoresceinated antigen-antibody complexes (F*[AgAb]) to define two distinct subpopulations of peripheral T cells, Fc receptor positive (Fc⁺) and Fc receptor negative (Fc⁻) T cells. Functional studies on populations of Fc⁻ and Fc⁺ T cells, purified on the fluorescence-activated cell sorter (FACS),[1] revealed that the Fc⁻ T-cell subset was not responsive to concanavalin A (Con A) and contained the helper T cells involved in collaboration with B cells in generation of humoral immune responses (16, 17). In contrast, the Fc⁺ T-cell subset was responsive to Con A and did not contain helper T-cell activity (16, 17). Both Fc⁺ and Fc⁻ T cells were directly stimulated by phytohemagglutinin (PHA).

The functional activity of T cells bearing the Fc receptor therefore remained to be found. However, the observed increases in Fc⁺ T cells as a result of allogeneic activation (18, 19) suggested that Fc⁺ T cells might be involved in cellular immune reactions. In the present report, we have examined the responsiveness of purified Fc⁻ and Fc⁺ T cells to alloantigens and have found that (a) both

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[1] Abbreviations used in this paper: Con A, concanavalin A; CL, cytotoxic lymphocyte; CML, cell-mediated lympholysis; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; MLR, mixed lymphocyte reaction; NMS, normal mouse serum; PHA, phytohemagglutinin.

subpopulations are capable of proliferating in response to allogeneic stimulation, (b) both subpopulations are involved in the generation of the cytotoxic response, and (c) the Fc^+ subpopulation, but not the Fc^- subpopulation, contains the cytotoxic effector cells.

Materials and Methods

Methods for preparation of antisera and cell suspensions, fluorescent staining procedures, analysis and separation of cells on nylon wool and on the FACS, and cell culture have been described in detail previously (16, 17). Anti-Ly-1.2 and Anti-Ly-2.2 were provided by Dr. Harvey Cantor and were prepared and absorbed as previously described (11). Complexes of fluoresceinated egg albumin and 7S BALB/cN anti-egg albumin were used throughout this study to label the Fc receptor (16).

Mice. Male and female mice from the inbred strains BALB/cN ($H-2^d$) and C57BL/10 ($H-2^b$) (with a champagne color mutation at the C locus) used in these experiments were obtained from our own colonies at Stanford.

Anti-Ly Cytotoxicity. Nylon purified C57BL/10 splenic T cells were pelleted and resuspended in a 1:20 dilution of anti-Ly serum (1 ml diluted antiserum per 2×10^7 cells) and incubated at 37°C for 20 min. Normal rabbit serum (as a source of complement) was then added to a final 1:12 dilution (90 μl per 1 ml of reaction mixture) and the incubation continued for an additional 30 min at 37°C. The cells were then centrifuged through a cushion of fetal calf serum (FCS, Grand Island Biological Co., Grand Island, N. Y.). Viability was determined by trypan blue dye exclusion.

Mixed Lymphocyte Reaction (MLR) and Cell-Mediated Lympholysis (CML). MLR and CML activity was determined by the method of Simpson et al. (20). Briefly, 5×10^5 BALB/cN lymphocytes were cultured with 5×10^5 (MLR) or 2.5×10^5 (CML) (1,500 R) syngeneic (BALB/cN) or allogeneic (B10) stimulators in 200 μl of RPMI-1640 (H-18, powder, Grand Island Biological Co.) supplemented to 10% vol/vol with FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO), 5×10^{-5} M 2-mercaptoethanol, and 10 mM HEPES buffer (no. 130440, GIBCO) in flat-bottomed Microtest II culture plates (Falcon Plastics, Oxnard, Calif.)

To determine MLR activity, 1 μCi of [^{3}H]thymidine (thymidine-[^{3}H]methyl, 6 Ci/mol, Schwarz/Mann Div. Becton, Dickinson & Co., Orangeburg, N. Y.) was added to each well after 3 days of culture. Culture was then continued for 8 h after which the cultures were harvested on a multiple automated sample harvester (MASH-II, Microbiological Associates, Bethesda, Md.) and counted on a Packard Tri-Carb Liquid Scintillation Spectrometer (Packard Instruments Co., Downers Grove, Ill.).

To determine CML activity, ^{51}Cr-labeled target cells (EL-4 or P815Y) were added to each well at the effector-to-target ratios specified in the Results section on the 5th day of culture. The plates were centrifuged for 5 min at 50 g and incubated at 37°C for 4 h. The plates were then centrifuged at 780 g for 15 min and 100-μl aliquots of supernate were collected from each well and counted in a gamma scintillation counter. Percent target lysis was determined by the formula:

$$\% \text{ lysis} = \frac{\text{cpm } ^{51}\text{Cr release} - \text{cpm spontaneous release}}{\text{cpm freeze/thaw release} - \text{spontaneous release}} \times 100$$

Preparation of Embryonic Fibroblast Monolayers. Fibroblast monolayers were prepared from 12–14-day-old mouse fetuses (21). The head, limbs, and tail were cut off each fetus and the liver extruded by pressing the belly with blunt forceps. The trunk was coarsely chopped with a razor blade and washed in 30–50 ml of Hanks' balanced salt solution (HBSS, GIBCO) for 10 min at room temperature, constantly stirred with a magnetic stirring bar. The fragments were then allowed to settle, and the supernate was decanted. The fragments were then incubated with 20 ml of trypsin-EDTA solution (0.5 g/liter trypsin and 0.2 g/liter EDTA in Puck's saline A, GIBCO) for 10 min at room temperature with constant stirring after which the supernate was again decanted. The fragments were then incubated for 20 min at room temperature with 20 ml of the trypsin-EDTA solution, after which the supernate was collected by filtration through nylon mesh screen. The supernate was centrifuged through a cushion of FCS at 380 g for 10 min, and the cells were resuspended to 10^5 cells/ml in RPMI-1640 supplemented to 10% with FCS and plated in 60-mm plastic culture plates (Falcon Plastics) at 10^6 cells per plate.
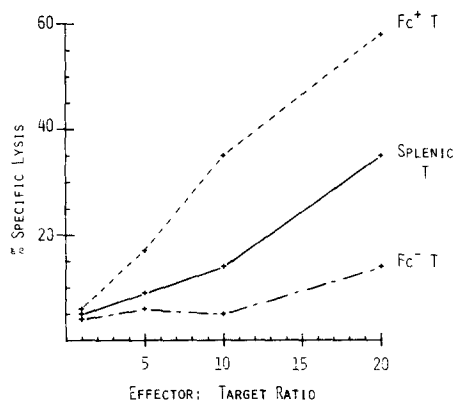
FIG. 1.   Cytotoxic activity of Fc⁺ vs Fc⁻ T lymphocytes isolated from *in vivo* sensitized mice. BALB/cN mice were sensitized by a single intraperitoneal injection of 10⁷ EL-4 tumor cells. Spleens were removed 12 days later, pooled, and separated on nylon wool and the FACS into Fc⁻ and Fc⁺ T-cell fractions. Each fraction was immediately assayed for lytic activity against ⁵¹Cr-labeled EL-4 target cells in vitro.

As soon as the monolayers reached confluency, they were gently washed 2 × with warm culture medium and overlaid with 10 ml of culture medium containing 10 × 10⁶ lymphocytes. At intervals thereafter, the lymphocytes were harvested from the plates by washing with warm medium, taking care not to disturb the fibroblast monolayer. The lymphocytes were washed once and assayed for their ability to bind antigen-antibody complexes.

Results

To determine whether the cytotoxic T lymphocytes (CL) from sensitized mice bear the Fc receptor, spleen cells, obtained from BALB/cN (H-2^a) mice 12 days after a single intraperitoneal injection of EL-4 tumor cells (H-2^b), were separated on nylon wool and the FACS into Fc⁻ and Fc⁺ T-cell subsets. The percent specific lysis of 5 × 10⁴ ⁵¹Cr-labeled EL-4 target cells was determined for each T-cell fraction at lymphocyte-to-target ratios of 1:1, 5:1, 10:1, and 20:1. As can be seen in Fig. 1, the Fc⁺ T-cell subset was substantially enriched in CL activity as compared to the unseparated splenic T-cell population. The Fc⁻ T-cell subset, on the other hand, displayed little or no CL activity.

It was previously suggested that the Fc receptor on T lymphocytes was acquired after allogeneic activation (18–19), implying that the precursors of cytotoxic lymphocytes were Fc⁻. To examine this hypothesis, Fc⁻ and Fc⁺ T lymphocytes, isolated from the spleens of normal (nonsensitized) BALB/cN mice, were cultured for 4–5 days with either syngeneic (BALB/cN) or allogeneic (B10) irradiated B cells. The ability to mount a proliferative response to the allogeneic cells was determined by pulsing the cultures, after 3 days of culture, with [³H]thymidine and assaying for [³H]thymidine incorporation. Parallel cultures were assayed after 5 days of culture for their ability to specifically lyse ⁵¹Cr-labeled EL-4 target cells to determine which cultures had contained the precursors of the cytotoxic effector cells.

Both the Fc⁺ (86% Fc⁺) and Fc⁻ (99% Fc⁻) fractions mounted comparable proliferative responses to the allogeneic cells (Fig. 2). The Fc⁺ T-cell fraction generated a cytotoxic response which was significantly higher than the response
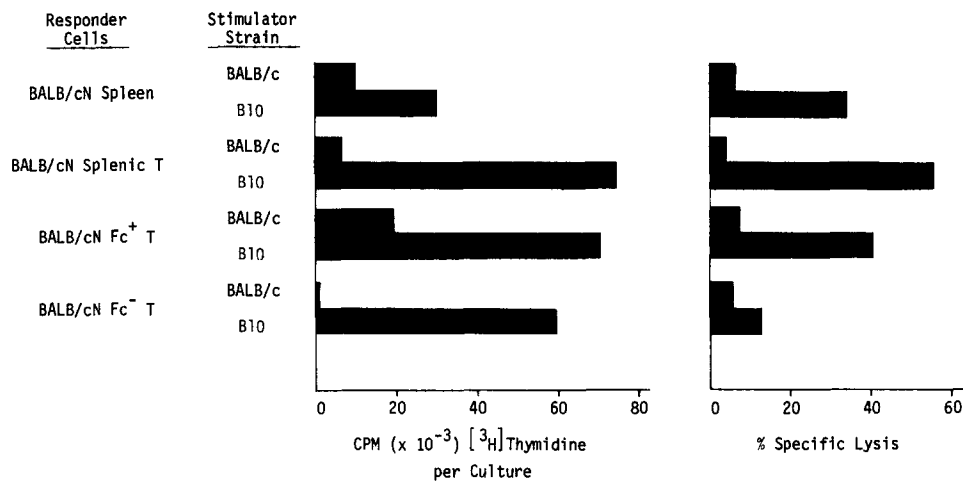
FIG. 2. MLR and CML activity of Fc⁻ and Fc⁺ T lymphocytes. Spleen cells from normal (nonsensitized) BALB/cN mice were separated on nylon wool and the FACS into Fc⁺ and Fc⁻ T-cell fractions. Each fraction was cultured at a concentration of 2.5×10^6 cells/ml (MLR) or 5×10^6 cells/ml (CML) along with an equal volume of irradiated B10 splenic B cells (2.5×10^6 cells/ml). Proliferative (MLR) responses were assayed by pulsing the cultures for 8 h with [³H]thymidine on the 3rd day of culture. Cytotoxic activity (CML) against ⁵¹Cr-labeled EL-4 target cells was assayed on the 5th day of culture at an effector-to-target ratio of 4:1.

generated by the Fc⁻ T-cell fraction (42 and 17% specific target cell lysis, respectively, at an effector-to-target ratio of 4:1). However, the cytotoxic responses generated by the Fc⁺ or Fc⁻ T-cell fractions did not exceed and were generally lower than the cytotoxic responses generated by unseparated splenic T cells (Fig. 2).

Since the unseparated T cells generated stronger cytotoxic responses than either the Fc⁺ or Fc⁻ T-cell fractions alone (Fig. 3a), it was considered possible that an interaction between the two populations was required for generation of a maximum cytotoxic response. To examine this possibility 2.5×10^5 irradiated B10 spleen cells were added to cultures containing either $1–5 \times 10^5$ Fc⁻ or Fc⁺ BALB/c splenic T cells, or mixtures of $1–5 \times 10^5$ Fc⁻ T cells plus the number of Fc⁺ T cells required to bring the number of responder T cells to 5×10^5 per culture. After 5 days of culture, the percent specific lysis per culture of 5×10^4 ⁵¹Cr-labeled EL-4 targets was determined. As can be seen in Fig. 3a, the cytotoxic responses generated by $1–5 \times 10^5$ splenic T cells exceeded the responses generated by $1–5 \times 10^5$ Fc⁺ or Fc⁻ T cells. The theoretical responses expected from mixtures of the Fc⁻ and Fc⁺ T cells, obtained by adding the responses obtained from equivalent numbers of Fc⁺ or Fc⁻ cells cultured alone, is shown in Fig. 3b. The responses actually obtained from cultures of these mixtures significantly exceeded the theoretical values. Thus, 1×10^5 Fc⁺ T cells generated a response of 3% target lysis when cultured alone, and 4×10^5 Fc⁻ T cells generated a response of 20% target lysis when cultured alone. When cultured together, 1×10^5 Fc⁺ cells and 4×10^5 Fc⁻ cells generated a response of 48% target lysis.
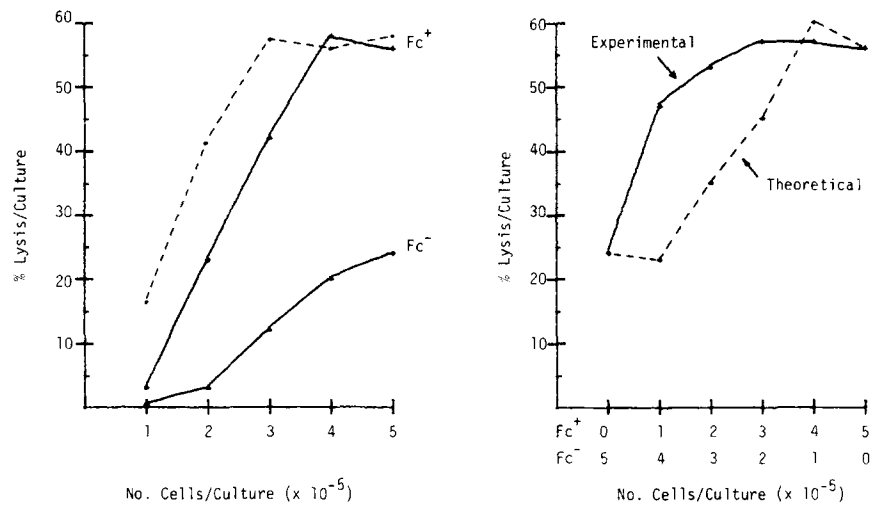
FIG. 3. Synergistic interaction between Fc⁺ and Fc⁻ splenic T cells in the generation of cytotoxic lymphocytes. Spleen cells from normal (nonsensitized) BALB/cN mice were separated on nylon wool and the FACS into Fc⁻ and Fc⁺ T-cell fractions. 1-5 × 10⁵ cells of each fraction were cultured with 2.5 × 10⁵ irradiated B10 splenic B cells in a total culture volume of 0.2 ml (Fig. 3a, left) or the Fc⁻ and Fc⁺ fractions were mixed in varying ratios, maintaining total cell number at 5 × 10⁵ responders per well (Fig. 3b, right). After 5 days of culture, the cytotoxic activity against 5 × 10⁴ ⁵¹Cr-labeled EL-4 targets was determined. Each point represents the arithmetic mean of three cultures.

To determine which T-cell fraction, Fc⁺ or Fc⁻, was contributing the precursors of the cytotoxic lymphocytes, C57BL/10 splenic T cells were treated with normal mouse serum (NMS), anti-Ly-1.2, or anti-Ly-2.2 plus complement and cultured overnight to clear aggregated Ig from the cell surface. Each fraction was then labeled with fluoresceinated antigen-antibody complexes and separated on the FACS into Fc⁺ or Fc⁻ fractions. The resultant T-cell fractions were cultured alone or mixed together for 5 days in the presence of 2 × 10⁵ irradiated BALB/cN spleen cells and assayed for their ability to lyse 3 × 10⁴ ⁵¹Cr-labeled P815Y tumor cells. As can be seen in Table I, treatment of splenic T cells with either Ly antiserum reduced the subsequent cytotoxic response. Mixture of the two Ly populations restored responsiveness to the level of untreated splenic T cells. Similarly, the Fc⁺ and Fc⁻ fractions of splenic T cells mounted weaker responses than the unseparated T cells. Mixtures of 1 × 10⁵ Fc⁺ T cells with 2 × 10⁵ Fc⁻ T cells responded as well as unseparated splenic T cells. Finally, mixtures of the Fc⁺ T cells separated from the Ly-1 T-cell subpopulation plus Fc⁻ T cells separated from the Ly-2 T-cell subpopulation responded as well as unseparated T cells, whereas the reciprocal mixture (Fc⁺Ly-2 + Fc⁻Ly-1) did not (Table I).

Although the above experiments indicated that the Fc⁺ and Fc⁻ T cells differed with respect to CL precursor and effector activity, it remained to be determined whether the proliferative response to allogeneic stimulation resulted in a loss or acquisition of Fc receptors. This question was approached by culturing nonsensitized, unseparated splenic T, Fc⁺ T, or Fc⁻ T cells on synge-

TABLE I

*Ly/Fc Phenotype of T Cells Synergizing in CML Responses**

Synergy group	Cell phenotype	No. cells/culture ($\times$ 10⁻⁵)	Lysis/culture‡
			%
Ly	Unseparated T	3	50 ± 5.2
	Ly-1⁺ T	3	16 ± 1.3
	Ly-2⁺ T	3	22 ± 1.5
	Ly-1⁺ T	1	47 ± 4.1
	Ly-2⁺ T	2	
Fc	Fc⁺ T	1	20 ± 3.1
	Fc⁻ T	2	14 ± 1.7
	Fc⁺ T	1	57 ± 6.1
	Fc⁻ T	2	
Fc/Ly	Ly-1/Fc⁺	1	43 ± 4.7
	Ly-2/Fc⁻	2	
	Ly-1/Fc⁻	1	18 ± 2.6
	Ly-2/Fc⁺	2	

* B10 splenic T cells were prepared by passage through nylon wool and treated with NMS, anti-Ly-1.2, or anti-Ly-2.2 plus complement. The cells were cultured overnight, subsequently stained with fluoresceinated antigen-antibody complexes, and separated on the FACS into Fc⁺ and Fc⁻ fractions. The fractions or combination of fractions were cultured 5 days with 2 $\times$ 10⁵ irradiated BALB/cN spleen cells.

‡ Percent specific lysis of 3 $\times$ 10⁴ ⁵¹Cr-labeled P815Y tumor cells. Average of triplicate cultures ± standard deviation.

neic (BALB/cN) or allogeneic (B10) embryonic fibroblast monolayers and assaying the T cells recovered from these cultures for the presence and surface density of Fc receptors at daily intervals after plating.

The fluorescence distribution of splenic T cells labeled with fluoresceinated antigen-antibody complexes is shown in Fig. 4 (day 0). At culture onset, 26% of the splenic T cells were Fc⁺, i.e. had a fluorescence intensity greater than 20 U (Fig. 5a), and this Fc⁺ subpopulation displayed a median fluorescence intensity (median density of Fc receptors per cell) of 183 U (Fig. 6a). As the interval between culture onset and assay increased, the intensity of labeling with fluoresceinated complexes increased (Fig. 4). The median fluorescence intensity per positive cell increased to 200 U after 4 days of culture on syngeneic fibroblasts and to 315 U after 4 days of culture on allogeneic fibroblasts (Figs. 4 and 6a). The proportion of splenic T cells binding 20 fluorescence U or more of complexes (Fc⁺) also increased during this period to 57% when cultured on allogeneic fibroblasts and to 39% when cultured on syngeneic fibroblasts (Fig. 5a).

Similar results were obtained when the separated Fc⁺ T cells were cultured on fibroblasts, although the difference between cells cultured on syngeneic as opposed to allogeneic fibroblasts was smaller (Fig. 7). At culture onset, 70% of the Fc⁺ cell fraction could be labeled with the fluoresceinated complexes (Fig. 5b). An increase in density of Fc receptors was observed as early as 1 day of

SPLENIC T CELLS (UNSEPARATED)

Day 0

Allogeneic Fibroblasts
- - - Syngeneic Fibroblasts

Day 2

Day 4

RELATIVE NUMBER OF CELLS

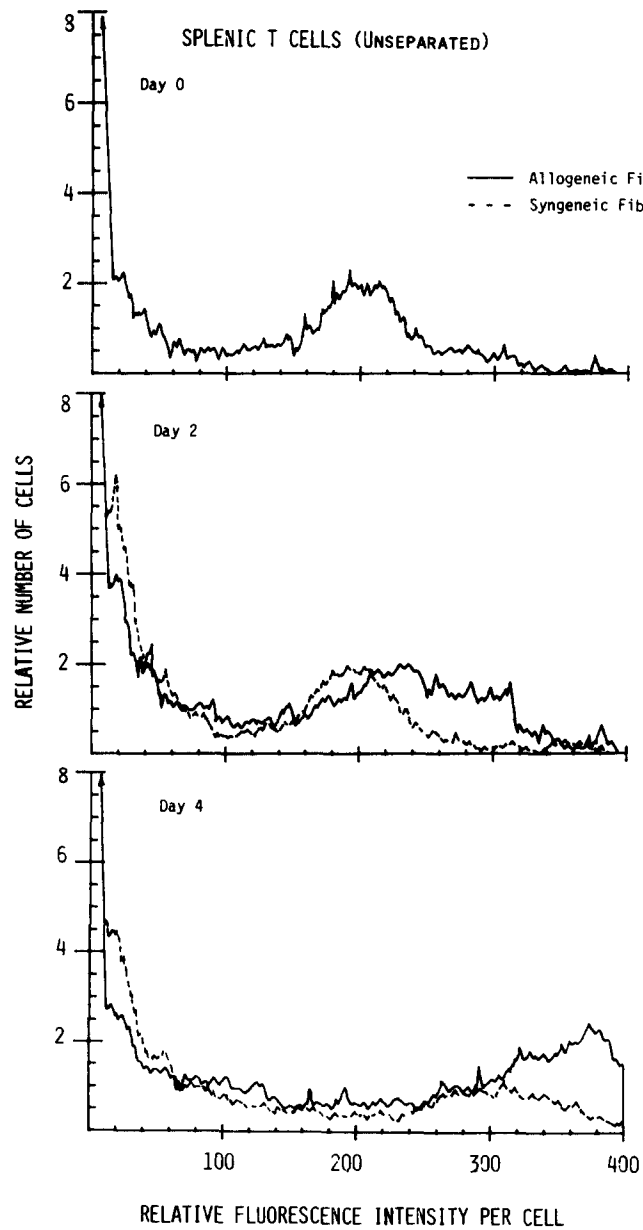100   200   300   400

RELATIVE FLUORESCENCE INTENSITY PER CELL

FIG. 4. Fluorescence distribution of splenic T cells labeled with fluorescent antigen-antibody complexes after activation on allogeneic monolayers. Nylon wool purified BALB/cN splenic T cells were cultured on B10 embryonic fibroblast monolayers. At time of culture onset (day 0), and after 2 or 4 days of culture, the T cells were harvested from the monolayer, labeled with fluorescent antigen-antibody complexes, and analyzed on the FACS. The distribution curves are based on cumulative analysis of 20,000 viable cells.

a) Splenic T Cells (unseparated)   b) Fc⁺ T Cells   c) Fc⁻ T Cells
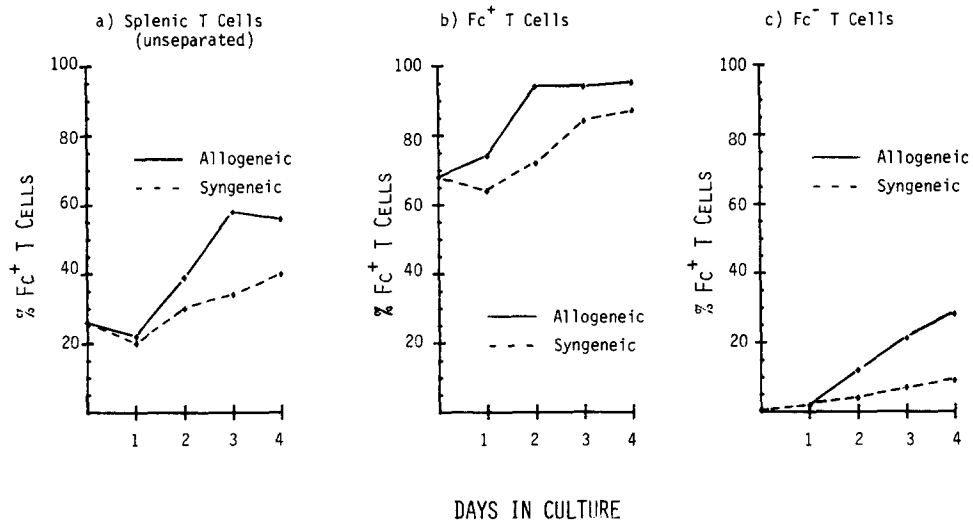
DAYS IN CULTURE

Fig. 5. Effect of allogeneic activation on the proportion of T cells binding antigen-antibody complexes. Nylon wool purified BALB/cN splenic T cells were cultured on B10 embryonic fibroblast monolayers. At time of culture onset (day 0), and after 2 or 4 days of culture, the T cells were harvested from the monolayer, labeled with fluorescent antigen-antibody complexes, and analyzed on the FACS. The proportion of Fc⁺ T cells is based on cumulative analysis of 20,000 viable cells.

a) Splenic T Cells (unseparated)   b) Fc⁺ T Cells   c) Fc⁻ T Cells
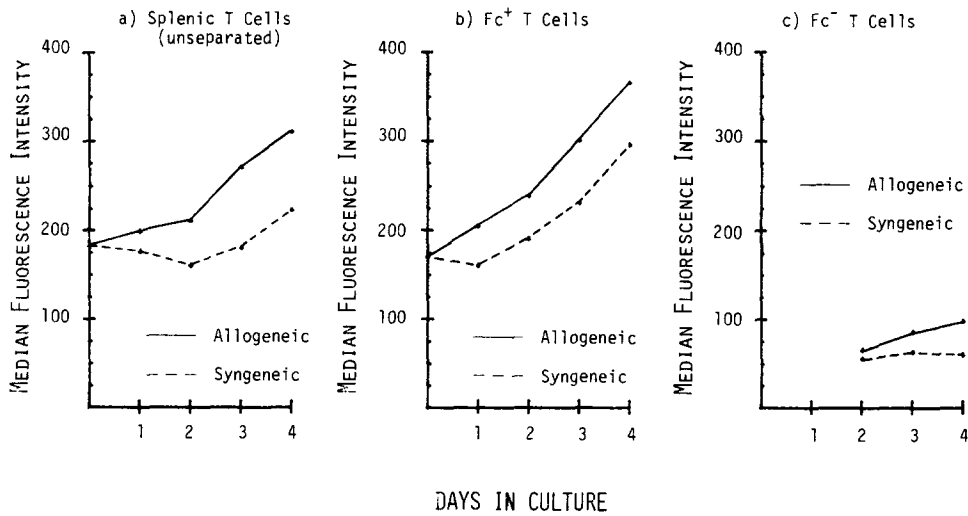
DAYS IN CULTURE

Fig. 6. Effect of allogeneic activation on the median fluorescence intensity of labeling with antigen-antibody complexes. Nylon wool purified BALB/cN splenic T cells were cultured on B10 embryonic fibroblast monolayers. At time of culture onset (day 0), and after 2 or 4 days of culture, the T cells were harvested from the monolayer, labeled with fluorescent antigen-antibody complexes, and analyzed on the FACS. The median fluorescence intensity was determined by FACS analysis of 20,000 viable cells and is defined as that level of fluorescence above which one-half of the positive (labeled) cells fluoresce.
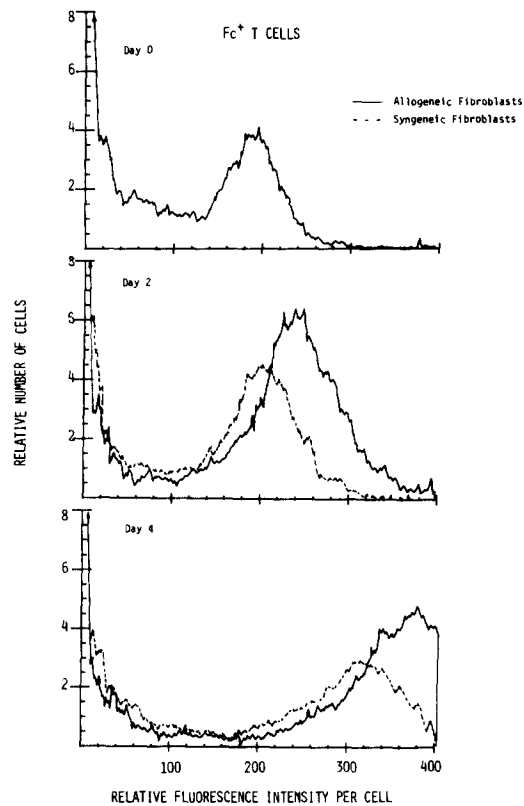
FIG. 7.   Fluorescence distribution of Fc⁺ T cells labeled with fluorescent antigen-antibody complexes after activation on allogeneic monolayers. Fc⁺ T cells obtained from normal BALB/cN mice were cultured on B10 embryonic fibroblast monolayers. At time of culture onset (day 0), and after 2 or 4 days of culture, the T cells were harvested from the monolayer, labeled with fluorescent antigen-antibody complexes, and analyzed on the FACS. The distribution curves are based on cumulative analysis of 20,000 viable cells.

culture on allogeneic fibroblasts (Fig. 6 b). The density of Fc receptors per cell continued to increase throughout the 4-day culture period, at the end of which greater than 90% of the cells cultured on allogeneic fibroblasts were Fc⁺ with a median fluorescence intensity of 370 U, and 87% of the cells cultured on synge-neic fibroblasts were Fc⁺ with a median fluorescence intensity of 290 U (Figs. 5 b and 6 b).

The fluorescence distribution of the Fc⁻ T-cell fraction labeled with fluores-ceinated complexes is shown in Fig. 8. At culture onset, only 0.1% of the Fc⁻ T cells labeled with fluoresceinated complexes (Fig. 5 c). Both the proportion of cells labeled and the median fluorescence intensity of labeling increased upon culture with allogeneic fibroblasts (Figs. 5 c, 6 c, 8). After 4 days of culture on allogeneic fibroblasts, 28% of the Fc⁻ T-cell fraction labeled with complexes (Fig. 5 c), but the median fluorescence intensity of this population (106 U) was significantly lower than the median intensity of the original Fc⁺ T-cell subset (170 U) before culture (cf. Fig. 6 b and c) and markedly lower than these cells after allogeneic culture (370 U).
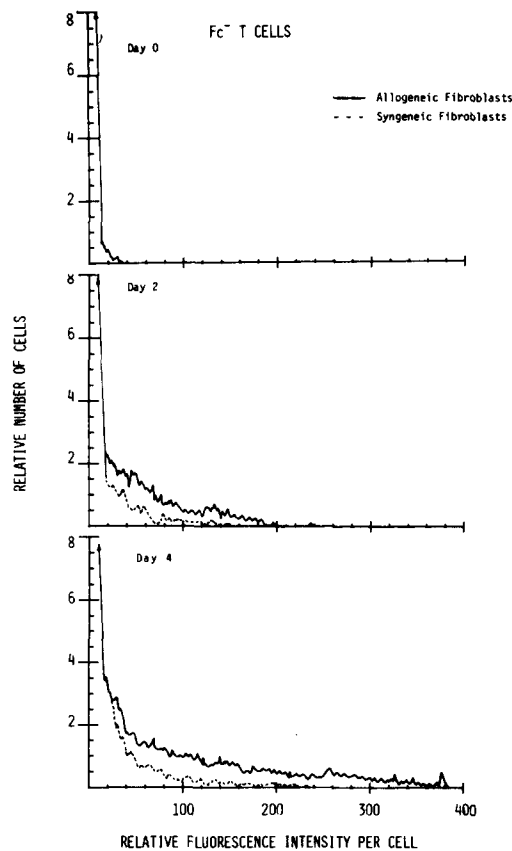
FIG. 8.   Fluorescence distribution of Fc⁻ T cells labeled with fluorescent antigen-antibody complexes after activation on allogeneic monolayers. Fc⁻ T cells obtained from normal BALB/cN mice were cultured on B10 embryonic fibroblast monolayers. At time of culture onset (day 0), and after 2 or 4 days of culture, the T cells were harvested from the monolayer, labeled with fluorescent antigen-antibody complexes, and analyzed on the FACS. The distribution curves are based on cumulative analysis of 20,000 viable cells.

Discussion

Utilizing the FACS, we have previously demonstrated that two distinct subpopulations of peripheral T cells could be defined on the basis of their ability to bind antigen-antibody complexes (16). The Fc⁻ T-cell subpopulation, but not the Fc⁺ T-cell subpopulation, contained the helper T cells involved in collaboration with B cells in the production of IgG antibody responses (16). The present report demonstrates that the Fc⁺ T-cell subpopulation, but not the Fc⁻ subpopulation, contains the cytotoxic effector lymphocytes generated by exposure to allogeneic cells and the amplifier T cells which play a collaborative role in generation of cytotoxic responses, thus establishing another functional distinction between the Fc⁻ and Fc⁺ T-cell subpopulations.

The Fc⁺ CL studied represented functionally sensitized cells insofar as separation into Fc⁻ and Fc⁺ T-cell fractions occurred after contact with antigen. Inasmuch as increases in the number of Fc⁺ T cells have been observed to be a

result of alloantigen activation (18, 19) the question arose as to whether the Fc receptor was acquired before or as a result of contact with antigen. When normal (nonsensitized) Fc^- or Fc^+ T cells were cultured with allogeneic lymphocytes, both the Fc^- and Fc^+ T cells mounted weaker cytotoxic responses than the unseparated splenic T cells. Cultures containing mixtures of the Fc^- and Fc^+ T cells mounted strong cytotoxic responses comparable to the responses of unseparated splenic T cells. Thus a strong cytotoxic response seems to require a synergistic interaction between the Fc^- and Fc^+ T cells similar to the synergistic interaction of $Ly-1^+$ and $Ly-2^+$ T lymphocytes reported by Cantor and Boyse (11, 22). In the latter system, it was demonstrated that (a) the precursors of CL were $Ly-2^+$ T cells, whereas the T cells responsible for amplification of CML were $Ly-1^+$, and (b) differentiation of $Ly-1^+$ cells from $Ly-2^+$ cells or vice versa did not occur. Ly phenotypes were therefore used to determine whether the CL precursors were Fc^- or Fc^+ T lymphocytes. Strong CML responses were obtained with mixtures of $Ly-2^+ Fc^-$ T cells plus $Ly-1^+ Fc^+$ T cells but not with $Ly-1^+ Fc^-$ T cells and $Ly-2^+ Fc^+$ T cells. It thus appears that the Fc^+ fraction of nonsensitized spleen cells contains the amplifier T cells, and the Fc^- fraction contains a CL precursor pool. Upon interaction with alloantigens the Fc^- T-cell precursors differentiate into Fc^+ cytotoxic lymphocytes. The ability of the Fc^+ fraction alone to generate significant CML responses may be due to the 10–15% Fc^- T cells which are present in the Fc^+ fraction after separation. Alternatively, it is possible that some of the CL precursors bear the Fc receptor.

Synergy between T cells in the generation of mature CL has been shown in a number of systems (1, 3, 4, 10, 11, 23, 24). As mentioned above, Cantor and Boyse (11) have shown that an $Ly-1^+$ T cell enhances the generation of CL from the $Ly-2^+$ CL precursor pool. It was not clear, however, whether this $Ly-1^+$ amplifier T cell simply amplified proliferation of the precursors during differentiation, thus increasing the number of CL derived from a given number of precursors, or whether the amplifier T cell was required for differentiation of CL to occur. Culturing Fc^- T cells, in the absence of the Fc^+ amplifier T cell, resulted in a significant increase in T cells which bind antigen-antibody complexes (Fig. 8). However, the presumably Fc^+ T cells derived in this manner bind antigen-antibody complexes to a considerably lower extent than either stimulated or unstimulated Fc^+ T cells (Fig. 7) and fail to display cytotoxic activity commensurate with the frequency of cells capable of binding antigen-antibody complexes (cf. splenic T vs. Fc^- T, Figs. 3a and 5). The failure of these cells to display significant cytotoxic activity suggests either that they represent an Fc^+ T-cell subset functionally distinct from the Fc^+ CL, that they were functionally impaired (paralyzed?) by exposure to antigen in the absence of amplifier T cells, or that they are functionally immature cells (e.g., that the acquisition of the Fc receptor may be an early differentiative event). Regardless of which alternative proves to be correct, it seems that the amplifier T cell is required for successful differentiation of the Fc^- CL precursors to Fc^+ cytotoxic effectors.

Two broad comparisons can be made between helper T-cell involvement in the differentiation of mature antibody-producing cells and amplifier T-cell involvement in the generation of CML responses. It has been demonstrated that

although B cells can react with a thymus-dependent antigen in the absence of helper T cells, the resultant antibody response is either absent (B-cell paralysis is induced) and or is limited to IgM production, i.e., maturation of IgG-producing cells does not occur (5, 6, 25–27). Thus, helper T cells seem to be required for some intermediate differentiative step of B-cell responses to antigen that leads to a classical IgG antibody response. Whether T-cell help is required for cells already committed (switched) to IgG or for switching cannot be specified at this time. Similarly, the Fc⁻ precursors of CL can react with alloantigens in the absence of amplifier T cells, as evidenced by their proliferation in mixed lymphocyte cultures (Fig. 2). As a result of this stimulation, a significant number of Fc⁺ T cells are generated. However, both the surface density of Fc receptors on these cells and the cytotoxic activity of these cells are very low. It may, therefore, be speculated that the amplifier T cell is required for an intermediate differentiative step in the T-cell response to alloantigens which leads to a classical cytotoxic (CML) response.

Secondly, it has been suggested that helper T-cell function may be associated with the *I*-region genes of the *H-2* complex in mice (28–29). We have recently demonstrated that antisera raised across *I*-region differences (anti-Ia sera) will block the ability of some (50–60%) of the Fc⁺ T cells to bind antigen-antibody complexes,[2] which suggests that a proportion of Fc⁺ T cells (the amplifier T cells?) express *I*-region-associated determinants. It is thus possible that amplifier T-cell function may be associated with the *I*-region of the *H-2* complex in mice (28, 29).

The similarities between helper and amplifier T cells discussed above raise interesting and, as yet unresolved, questions concerning the membrane markers used to identify them. Both helper T cells and amplifier T cells bear the Ly-1 determinant but not the Ly-2 or Ly-3 determinants (22). Is the Ly-1 determinant expressed in the absence of Ly-2 or Ly-3 exclusively by cells whose function is to collaborate in the differentiation of effector lymphocytes, and, if so, is the Ly determinant relevant to this function? We have now demonstrated in this and preceding reports (16, 17), that the amplifier T cells and helper T cells represent distinct subpopulations of T cells, insofar as the helper T cells are Fc⁻ and the amplifier T cells are Fc⁺. Elucidation of the functional relevance of the Fc receptor may allow discernment of the functional distinction between helper and amplifier T cells.

It may be that some functional overlap exists between the amplifier T cell and the helper T cell, or alternatively, that interaction between the two T cells can occur. We have previously demonstrated that the Fc⁺ T cells are responsible for the mitotic response to Con A, and, that as a result of Con A stimulation, the Fc⁺ T cells could "recruit" Fc⁻ T cells into the mitotic response (17). In the absence of Fc⁺ T cells, Fc⁻ T cells did not respond well to Con A. Hirst et al. (30) have reported that an Ly-1⁺ cell is required for Con A stimulation, implicating an Fc⁺/Ly-1 T cell (amplifier T?) in the Con A response. Waterfield et al. (31) have reported that culturing T cells with Con A results in the appearance of cytotoxic

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[2] Stout, R. D., D. B. Murphy, H. O. McDevitt, and L. A. Herzenberg. The Fc receptor on thymus-derived lymphocytes. IV. Inhibition of binding of antigen-antibody complexes to Fc⁺ T cells by anti-Ia antisera. Manuscript in preparation.

effector cells. It is thus possible that nonspecifically activated amplifier T cells can drive the differentiation of precursors into cytotoxic effectors. It has also been reported that activation of T cells by Con A (32) or by allogeneic stimulation (33) can nonspecifically enhance the generation of antibody-forming cells. Thus, it would seem that activated amplifier T cells either can enhance B-cell responses directly (e.g., there is a functional overlap between amplifier and helper T cells) and/or can enhance the generation of functional helper T cells from nonsensitized T-cell populations.

In conclusion, it is worth stating concisely the observations on T-cell heterogeneity inherent in the data presented in this and preceding reports (16, 17). There appear to be at least three functionally distinct subsets of T lymphocytes, Fc^-/ Ly-1 helper T, Fc^+/Ly-1 amplifier T, and Fc^-Ly-2 CL progenitor T, which may arise through distinct differentiation pathways before exposure to antigen. Examination of the Ly antigens expressed by T cells from normal, nonsensitized mice has confirmed that a significant proportion of Ly-1 and Ly-2 T cells bear the Fc receptor as detected by our labeling procedure and that a significant proportion lack this Fc receptor.[3] We are presently investigating the differentiation, function, and intercellular interactions of T-cell subsets separated on the basis of their expression of select surface membrane markers, including the Fc receptor and Ly antigens.

Summary

The involvement of Fc^- and Fc^+ T cells, separated on the fluorescence-activated cell sorter, in proliferative and cytotoxic responses to alloantigens was examined. The cytotoxic lymphocytes generated by in vivo exposure to allogeneic tumor cells were shown to express the Fc receptor. The proliferative responses to alloantigen exposure in mixed lymphocyte cultures was equivalent in intensity for unseparated T cells, the Fc^+ T-cell fraction, and the Fc^- T-cell fraction isolated from nonsensitized spleen cells. In contrast, the cytotoxic responses generated by the Fc^- T-cell fraction ($<1\%$ Fc^+) were much weaker than the cytotoxic responses generated by the Fc^+ T-cell fraction (80–90% Fc^+), and the responses of the Fc^+ T-cell fraction were generally weaker than, or equal to, the responses of unseparated T cells (Fc^- T $\ll$ Fc^+ T $\leq$ unseparated T). Mixtures of the Fc^- and Fc^+ T-cell fractions mounted stronger cytotoxic responses than the sum of the responses of either fraction alone. Examination of the Ly phenotypes of the synergizing populations revealed that the CL precursor activity (Ly-2^+ T cells) resided in the Fc^- T-cell population, and that the amplifier T-cell activity (Ly-1^+ T cells) resided in the Fc^+ T-cell population. The data are discussed in terms of T-cell heterogeneity, differentiation, and intercellular interaction.

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[3] Stout, R. D., H. Cantor, F. W. Shen, E. A. Boyse, and L. A. Herzenberg. 1976. The Fc receptor on thymus-derived lymphocytes. V. Distinct subsets within Fc^- and Fc^+ T cells defined by their expression of Thy 1 and Ly antigens. Manuscript in preparation.

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