THE Fc RECEPTOR ON THYMUS-DERIVED LYMPHOCYTES*

I. Detection of a Subpopulation of Murine T Lymphocytes Bearing the Fc Receptor

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Studies in several laboratories have shown that mammalian lymphocytes bear on their surface membranes a receptor which specifically binds aggregated immunoglobulin (1–6). These studies have shown that the receptor (termed the Fc receptor) is distinct from the complement receptor, is specific for a site on the Fc portion of the aggregated immunoglobulin, and is present predominantly on B lymphocytes (1–6).

Recently, several reports have suggested that the Fc receptor may be present on activated T lymphocytes in addition to B lymphocytes (7–9). The functional significance of the Fc receptor on activated T cells, however, remains unclear. Experiments were initiated in this lab to examine normal murine lymphocytes bearing the Fc receptor, taking advantage of the fluorescence-activated cell sorter (FACS)[1] and its ability to provide independent volume and quantitative fluorescence measurements of individual cells as well as its ability to provide highly purified, functionally viable populations of the labeled cells. The present study describes a significant subpopulation of normal T lymphocytes which bear the Fc receptor and demonstrates that this Fc^+ T-cell subpopulation does not include the antigen-specific "helper" T cell.

Materials and Methods

Mice. Male and female mice from inbred strains of BALB/cN and (SJL × BALB/cN)F_1 used in these experiments were obtained from our own colonies at Stanford.

Antisera. A rabbit antiserum prepared against a saline EDTA extract of rabbit antimouse thymocyte (RAMT) and absorbed with mouse bone marrow was shown to be specific for T cells (10). Rabbit antimouse Ig (RAMIG) was purified by repeated injections of rabbits with ammonium sulfate prepared mouse Ig in complete Freund's adjuvant. Goat antirabbit Ig(GARIG) was prepared by repeated injections of goats with DEAE-cellulose purified rabbit immunoglobulin in complete Freund's adjuvant. Anti-Thy 1.2 (antitheta) was prepared by Dr. G. Michael Iverson from ascites fluid elicited by intraperitoneal injection of Sarcoma 180 cells into AKR mice after immunization with CBA thymocytes according to the method of Reif and Allen (11). BALB/cN

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; D-PBS, Dulbecco's phosphate-buffered saline; F*(AgAb), fluoresceinated antigen-antibody complexes; FACS, flourescence-activated cell sorter; FCS, fetal calf serum; GARIG, goat antirabbit Ig; KLH, keyhole limpet hemocyanin; OV, ovalbumin; PFC, plaque-forming cell; RAMIG, rabbit antimouse Ig; RAMT, rabbit antimouse thymocyte.

antikeyhole limpet hemocyanin (KLH, Pacific Biomarine, Venice, Calif.) or antiegg albumin (OA, Miles Laboratories, Inc., Kankakee, Ill.) was prepared by intraperitoneal injection of 100 μg alum-precipitated KLH into 6- to 8-week old BALB/cN mice. 4 wk later the mice were boosted with 100 μg aqueous KLH administered intravenously. The mice were bled 7 and 10 days after the boost. The sera were pooled, ammonium sulfate precipitated, and the 7S fraction purified by chromatography on Sephadex G-200. $F(ab')_2$ fragment of the 7S Sephadex G-200 fraction was prepared by pepsin digestion (1:100 ratio pepsin to protein) at 37°C for 20 h in Walpole's acetate buffer, pH 4.2 (12) and subsequent separation on Sephadex G-200.

All serum preparations were deaggregated by centrifugation at 80,000*g* for 1 h, sterilized by passage through a 0.22 μm Millipore filter, and stored at 4°C. Fluorescein conjugates of the ammonium sulfate-purified Ig of GARIG and AKR anti-Thy 1.2 were prepared according to the method of Cebra and Goldstein (13).

Cell Suspensions. Mice were sacrificed by cervical dislocation and the spleens, thymii, and peripheral lymph nodes excised. Cell suspensions were prepared by cutting the organs into small fragments and gently pressing them between two glass slides in Dulbecco's phosphate-buffered saline (D-PBS) (14) supplemented to 5% vol/vol with fetal calf serum (FCS). All cell suspensions were dispersed by aspiration with a Pasteur pipette. Connective tissue and tissue clumps were eliminated by passage through nylon screen. Erythrocytes were eliminated by treatment with NH_4Cl buffered in Gey's solution.

Cell Transfer for Adoptive Anti-DNP Responses. The complete methodology for adoptive transfer and subsequent plaque assay has been published previously (15). Briefly, splenic B lymphocytes, prepared from spleens of DNP-KLH primed mice by treatment with anti-Thy 1 plus complement, were transferred to lethally irradiated syngeneic recipients along with nylon wool-purified splenic T cells and 100 μg aqueous DNP-KLH. The recipient spleens were removed 7 days later and assayed for direct and indirect anti-DNP PFC by a modification of the plaque assay described by Cunningham and Szenberg (16).

Fluorescence Staining of Cells. Pelleted cells (1×10^7) were resuspended in 0.1 ml of the labeling reagent (0.5 mg/ml RAMT, 1.2 mg/ml RAMIG, 0.5 mg/ml fluoresceinated anti-Thy 1.2) and left at room temperature for 20 min. The cell suspension was pelleted through FCS and subsequently washed in 2 ml of medium. Except in the case of directly fluoresceinated anti-Thy 1.2, the cell pellets were resuspended in 0.1 ml of fluoresceinated GARIG at 0.4 mg/ml and left at room temperature for 20 min. The cells were washed as above, resuspended to $2–5 \times 10^6$ cells/ml in D-PBS, and held on ice for analysis.

Labeling of Fc Receptor. Fluoresceinated antigen-antibody complexes were prepared by adding a 10- to 20-fold excess of 7S anti-KLH to fluoresceinated KLH, incubating 2 h at 37°C, and then overnight at 4°C. The fluoresceinated antigen-antibody complexes (F*(AgAb)) were pelleted by centrifugation at 30,000*g* and resuspended in D-PBS to 1 mg protein per ml. Pelleted cells (1×10^7) were resuspended in 0.1 ml of the fluoresceinated complexes and incubated at 37°C for 30 min. The cells were pelleted through 1 ml of FCS and subsequently washed in 2 ml of D-PBS.

Fluorescence Microscopy. A Zeiss microscope with an HB200 mercury arc (OSRAM) light source was used (Carl Zeiss, Inc., New York). Cells were examined with dark-field illumination using a Zeiss 1.2/1.4 NA oil immersion planochromat objective with an iris diaphragm. Combination of a Zeiss PIL 546 NM excitation filter and two layers of Kodak Wratten no. 23A gelatin barrier filter (Eastman Kodak Co., Rochester, N. Y.) were used to detect fluorescence.

Cell Separation. Immunoglobulin-bearing cells were removed from spleen cell suspensions by passage through nylon wool according to the method of Julius et al. (17). Briefly, 0.6 g nylon wool (LP-1) Leuko-Pak leukocyte filters (Fenwal Laboratories, Morton Grove, Ill.) were packed into the barrel of a 12 ml syringe up to the 6 ml mark and thoroughly wetted with D-PBS. The column was incubated at 37°C for at least 1 h and washed with 50–80 ml of warm (37°C) D-PBS. The columns were sealed with parafilm and incubated at 37°C for 45 min. The column was then washed slowly with 24 ml of warm D-PBS and the effluent, which contained a highly pure population of T cells, (Table II), collected. The nylon wool was then removed from the column, eluted with 50–80 ml of cold (4°C) D-PBS, and the eluate, which was enriched for Ig-bearing cells (Table II), was collected. Viability, as determined by trypan blue dye exclusion, was usually greater than 90%.

Cells labeled with fluoresceinated anti-Thy 1.2 or AgAb were separated from unlabeled cells using a FACS as previously described (18–20). In the latest version, FACS-1, (made by Becton Dickinson Electronics Laboratory, Mountain View, Calif.) of this instrument, cells were observed

individually in suspension in the central stream of a 70 μm diameter coaxial liquid jet as it passes through a laser beam. One photo detector system provides a measure of cell size by detecting light-scattering characteristics and a second photo detector system provides a quantitative measure of fluorescence intensity using appropriate optical filters. The liquid jet is later broken into uniform-sized droplets, and those droplets containing the desired cells are charged electrically and deflected in an electric field. The cell populations separated in these experiments were processed at a rate of 5,000 viable cells per second, which permitted a substantial enrichment of positive fractions and virtually 100% pure negative fractions (see Table I).

Analysis of Cell Populations on the FACS. Analysis of cell populations on the FACS involved the same principles as involved in cell separation described above. Cells were processed at 500–1,000 cells per second and the intensity of fluorescence (pulse height) was recorded for each individual cell on the pulse height analyzer. The level of background fluorescence was determined by analyzing appropriate negative controls (e.g., cells labeled with normal rabbit serum). Thus, the percentage of labeled cells was obtained by counting the number of cells giving fluorescence signals above background and dividing by the total number of viable cells examined. Light-scattering signals distinguish live from dead lymphocytes (20). This calculation was performed by the FACS analyzer and was based on analysis of 5-10 $\times$ 10^4 individual viable cells.

Results

Exposure of single cell suspensions of spleen from 12-wk old BALB/cN mice to 200 μg of fluoresceinated KLH-anti-KLH complexes (F*(AgAb)) resulted in labeling of approximately 65% of the cells (Fig. 1). This level of labeling represents saturation of the binding cell population insofar as further increases in concentration of F*(AgAb) did not increase the number of spleen cells labeled. At saturation (200 μg F*(AgAb)), the fluorescence distribution of the labeled spleen cells (i.e., the fluorescence intensity per cell) was very broad, indicating a large variation in the amount of F*(AgAb) bound per cell in the splenic lymphocyte population (Fig. 2)

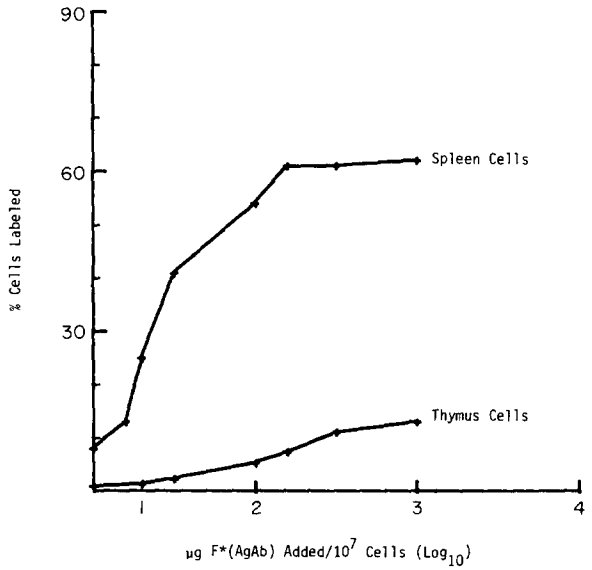

FIG. 1. Optimal concentration of F*(AgAb) for labeling of spleen and thymus cell populations. 10 million spleen or thymus cells were incubated 30 min at 37°C with 10–1,000 μg of F*(AgAb), washed through FCS, and analyzed on the FACS.

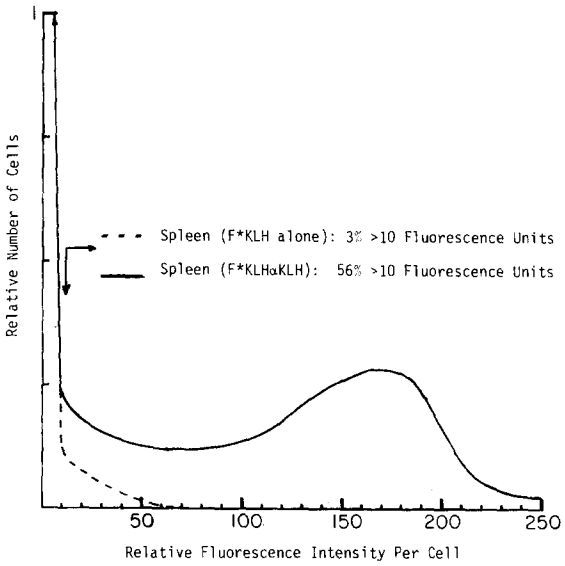

FIG. 2. Fluorescence distribution of spleen cells labeled with F*(AgAb). 10 million spleen cells were incubated 30 min at 37°C with 200 μg of F*KLH or 200 μg of F*KLH anti-KLH complexes, washed through FCS, and analyzed on the FACS. The distribution curve is based on cumulative analysis of 25,000 viable cells.

To establish the specificity of the binding of F*(AgAb) to spleen cells, complexes were prepared with F*KLH and 19S (IgM) anti-KLH, 7S anti-KLH, or $F(ab')_2$ anti-KLH. Exposure of spleen cells to 200 μg of complexes prepared with 7S anti-KLH resulted in labeling of 60% of the cells (Table I). Similar exposure of spleen cells to 200 μg of complexes prepared with either 19S anti-KLH or $F(ab')_2$ anti-KLH resulted in labeling of less than 5% of the cells. Complexes prepared with fluoresceinated egg albumin (F*OA) and 7S anti-OA labeled the splenic lymphocytes to the same extent as complexes prepared with F*KLH and 7S anti-KLH (Table I). Thus, the labeling of spleen cells with F*(AgAb) was due to binding of the Fc portion of 7S antibody and was independent of the antigen binding specificity of that antibody.

The number of Fc^+ cells in spleen consistently exceeded the number of Ig-bearing cells (54% and 43%, respectively, in Table II). To determine the identity of these Fc^+ Ig^- cells, spleen cells, before and after separation on nylon wool, were stained for membrane Ig (RAMIG), T-cell markers (RAMT), and/or the Fc receptor. In the intact spleen cell population, 9% of the cells were labeled by both the anti-T cell (RAMT) and the F*(AgAb) reagents (Table II). In the effluent fraction of the nylon wool separated spleen cells, 98% of the cells were labeled with RAMT, 18% of which were labeled with F*(AgAb) (Table II). Similar results were obtained using an AKR anti-CBA Thy 1.2 serum instead of RAMT. When the amount of F*(AgAb) bound per cell (fluorescence distribution) by the purified splenic T lymphocytes was compared with that bound per cell by the population enriched for splenic lymphocytes (nylon wool effluent and eluate, respectively, Table II), no significant difference was observed (Fig. 3). Although the number of labeled cells was higher in the B-cell-enriched population, both

TABLE I
Specificity of the Fc Receptor for 7S Antibody

Labeling reagent*	Spleen cells labeled‡
	%
F*KLH	1.8 ± 0.8
(F*KLH + 19SαKLH)	4.0 ± 2.0
(F*KLH + 7SαKLH)	58 ± 7
(F*KLH + F(ab')$_2\alpha$KLH)	4.2 ± 2.3
(F*OA + 7SαOA)	62 ± 5

* 10^7 Spleen cells were incubated 30 min at 37°C with 200 μg of indicated complexes, washed through FCS, and analyzed on the FACS.
‡ Numerical values represent arithmetic mean of five experiments ± 1 SD.

TABLE II
Presence of Fc Receptor on Peripheral T Cells

Cells	Cells*			
	Ig^+	T^+	Fc^+	T^+ and Fc^+ (Doubles)
	%		%	
Unseparated spleen	43	41	54	9
Nylon wool effluent	1	98	18	16
Nylon wool eluate	65	25	78	8

* Cells were labeled with RAMIG or RAMG followed by rhodaminated G or RIG ± F* (AgAb) or with F* (AgAb) alone. Percentage of cells labeled is based on microscope analysis of 200–400 cells.

cell populations displayed cells labeling with the complexes over a broad range of fluorescence intensity.

The tissue distribution of the Fc^+ lymphocytes was examined by labeling lymphocytes from spleen, peripheral lymph nodes, and thymus with RAMT (rhodamine) and F*(AgAb) (fluorescein) and counting the number of cells bearing both labels under a fluorescent microscope. As can be seen in Table III, the percentage of T lymphocytes bearing Fc receptor was approximately the same (~23%) in spleen and lymph node. Thymus contained a smaller subpopulation (~10%) of cells bearing the Fc receptor (Table III and Fig. 1).

To investigate the previously proposed hypothesis (8) that the Fc^+ T lymphocytes are antigen-activated T cells (e.g., lymphoblasts and/or cooperator T cells), the size distribution of the Fc^+ splenic T cells (those T cells having fluorescence intensity greater than 10 U as displayed in Fig. 3) was compared with the size distribution of the Fc^- T cells (those T cells having a fluorescence intensity of less than 10 U). As can be seen in Fig. 4, the size distribution of the Fc^+ and Fc^- T cells were essentially similar—both populations displayed a predominance (>70%) of small cells with a minor (13% of Fc^- and 28% of Fc^+) population of

TABLE III
Frequency of Fc^+ T Lymphocytes in BALB/cN Lymphoid Tissue

Tissue	Fraction of labeled cells*			
	T^+/total	Fc^+/total	Fc^+T^+/total	Fc^+T^+/T^+
Spleen	0.46 ± 0.08	0.61 ± 0.10	0.11 ± 0.04	0.24
Lymph node	0.75 ± 0.09	0.38 ± 0.08	0.16 ± 0.06	0.22
Thymus	0.97 ± 0.02	0.10 ± 0.04	—	—

* Cells were labeled with RAMT followed by rhodaminated GARIG plus F* (AgAb). Fraction of cells labeled is based on microscope analysis of 200–500 cells. Numerical values represent arithmetic mean ± SD of three experiments.

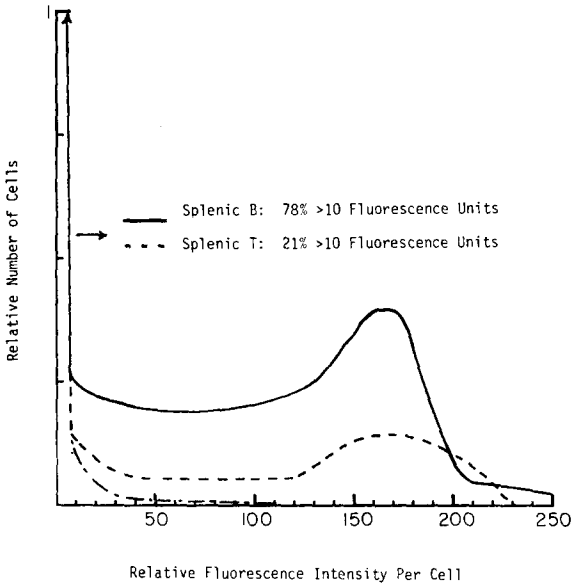

FIG. 3. Fluorescence distribution of nylon wool purified splenic B and T cells labeled with F*(AgAb). 10 million nylon wool purified splenic B cells (69% Ig^+) or splenic T cells (<1% Ig^+) were incubated 30 min at 37°C with 200 μg F*(AgAb), washed through FCS, and analyzed on the FACS. The distribution curves are based on cumulative analyses of 25,000 viable cells.

large cells (those cells having a size greater than 135 U as displayed in Fig. 4).

To determine whether the Fc^+ T lymphocytes are capable of cooperating with B lymphocytes in the humoral immune response to DNP-KLH, splenic T cells from (SJL × BALB)F_1 mice primed 1 wk previously with 100 μg alum-precipitated KLH were purified by nylon wool filtration, labeled with complexes of F*OA-7S anti-OA, and separated on the FACS into Fc^+ (23% of T cells) and Fc^- (77% of T cells) fractions. Graded numbers of these 1 wk KLH-primed T lymphocytes were then transferred into lethally irradiated syngeneic recipients along with 100 μg DNP-KLH and 5 × 10^6 anti-theta plus complement-treated spleen cells from (SJL × BALB)F_1 mice primed 1 mo previously with 100 μg alum-

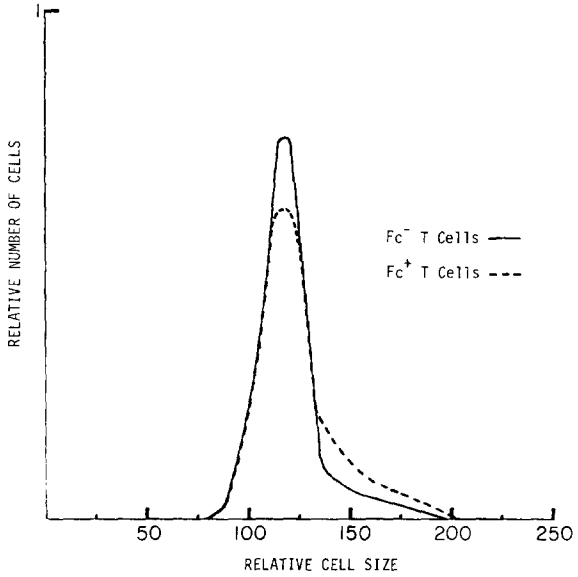

FIG. 4. Size distribution of Fc^+ vs. Fc^- splenic T cells. 10 million nylon wool purified splenic T lymphocytes (<1% Ig^+) were incubated 30 min at 37°C with 200 μg F*(AgAb), washed through FCS, and analyzed on the FACS. The size distributions of 25,000 cells labeling with a fluorescence intensity of greater than 10 U (Fc^+ T, as shown in Fig. 3) or less than 10 U (Fc^- T) are represented.

precipitated DNP-KLH (DNP-primed splenic B cells). 1 wk later, the recipient spleens were assayed for direct and indirect DNP specific PFC. As can be seen in Fig. 5, recipients of 5×10^6 DNP primed splenic B cells without KLH-primed T lymphocytes responded very poorly (<50 indirect DNP-PFC/10^6 spleen cells). This response increased linearly with increasing doses of KLH-primed T cells up to 5,100 indirect DNP-PFC/10^6 spleen cells of recipients receiving 3×10^6 unseparated T cells. Recipients of 0.75×10^6 Fc^+ T cells, which constituted 23% of the splenic T-cell population and therefore were equivalent to 3×10^6 unseparated T cells, did not mount a significant indirect DNP-PFC response (300 PFC/10^6 recipient spleen cells). Recipients of 1.5×10^6 Fc^- T cells responded as well as recipients of 1.5×10^6 unseparated T cells—3,116 DNP-PFC/10^6 cells and 2,700 PFC/10^6 cells respectively. The same result was obtained when splenic T cells from donors primed with KLH 1 mo previously were used.

Discussion

Utilizing the FACS and murine antibody-antigen complexes formed in antibody excess, we have demonstrated the presence of the Fc receptor on the surface of a distinct subpopulation of murine T lymphocytes. The fluorescence distributions of F*(AgAb)-labeled B and T cells were both very broad, indicating a large variation in the amount of complexes bound per cell. This lack of uniformity of staining is in accord with the great heterogeneity in the number of Fc receptors per cell reported by Andersson and Grey (9). The fluorescence distributions of the labeled T- and B-cell populations were almost completely overlapping. Thus, there appeared to be no significant difference in the amount

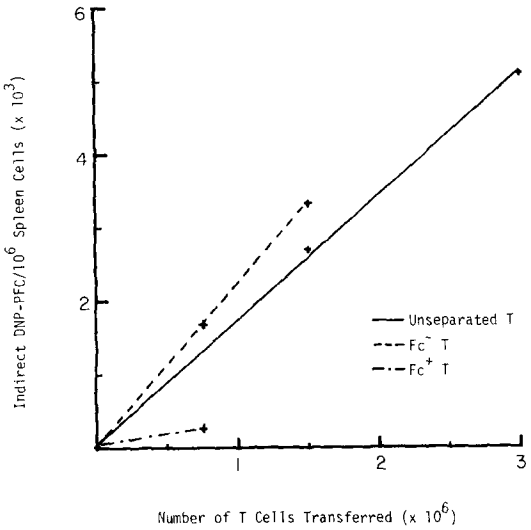

FIG. 5. Cooperator activity of Fc^+ vs. Fc^- splenic T cells. Splenic T cells were prepared by nylon wool filtration of spleen cells from mice primed 1 wk previously with 100 μg KLH. 100 million T cells were labeled with 2 mg F*OA anti-OA complexes and separated into Fc^+ (fluorescence intensity greater than 10 U, Fig. 3) and Fc^- (fluorescence intensity less than 10 U, Fig. 3) on the FACS. Graded numbers of these T cells ($0–3 \times 10^6$) were transferred into lethally irradiated recipients along with 100 μg DNP-KLH and 5×10^6 antitheta-treated DNP-KLH-primed splenic B cells. 1 wk later the recipient spleens were pooled and assayed for direct and indirect DNP-PFC. The number of indirect DNP-PFC per 10^6 spleen cells are presented as a function of number T cells transferred.

of complexes bound per cell between the peripheral Fc^+ T and B lymphocytes.

Several groups have recently reported that the Fc receptor appears on T lymphocytes activated by exposure to KLH (8) or allogeneic cells (7, 9). We have also found an increase in the number of Fc^+ T cells as a result of allogeneic activation (unpublished observation). The data reported here, however, suggest that the state of activation and the presence of the Fc receptor on T lymphocytes are not strictly correlated. First, the majority (70%) of Fc^+ T cells in normal spleen cell populations are small lymphocytes (e.g., not lymphoblasts). This indicates that the T lymphocytes do not need to be in an activated state to express the Fc receptor. Secondly, only 50–60% of the large "activated" T cells bear the Fc receptor (7–9), suggesting that activation does not always lead to acquisition of the Fc receptor. Finally, it was reported that KLH-activation of T lymphocytes resulted in a substantial increase in Fc^+ T cells (8). However, as shown in this study, the Fc^+ T lymphocytes isolated from spleen 1 wk after priming with KLH did not cooperate with DNP-primed B lymphocytes to give an adoptive PFC response to DNP-KLH. Rather, all of the T-cell helper activity, on a per cell basis, was recovered in the Fc^- T-cell population. Thus, we find that the reported increase in Fc^+ T lymphocytes upon antigen-activation appears to be irrelevant to the functional (cooperator) activation which occurred.

The question thus arises as to why activation leads to an increase in Fc^+ lymphocytes and/or what the function of the Fc^+ T lymphocytes is. The lack of cooperator activity of Fc^+ T cells isolated from mice 1 wk or 1 mo after carrier

priming makes it unlikely that the Fc receptor is a general marker for primed T cells. However, due to the observed increase in numbers of Fc^+ T cells upon exposure to activating conditions, we cannot exclude the possibility that the Fc receptor may be a marker for differentiation in subsets of T cells other than the cooperator T cells (21, 22).

Cantor et al. have described two T-cell subsets which can be distinguished on the basis of Thy 1.2 density (22) and anatomical localization (23), one subpopulation (T_1) being primarily restricted to thymus and spleen and the second subpopulation (T_2) being primarily restricted to lymph node and blood. Since an equivalent proportion of the T cells in lymph node and spleen bear the Fc receptor (Table III) it is unlikely that the Fc^+ T cell is restricted to either the T_1 or T_2 subpopulations.

Gorczynski et al. (24) have recently attempted to explain the bulk of immunosuppression phenomena in terms of blocking of lymphocyte function by antigen-antibody complexes. Their arguments are based on two hypotheses: first, that an Fc receptor exists on T lymphocytes; and second, that the Fc receptor or the cell bearing that receptor exerts a control function. The presence of an Fc receptor on a distinct subpopulation of murine T lymphocytes has been demonstrated in this report, thus confirming their first hypothesis. Their second hypothesis would predict that the Fc^+ T cells may be active in one or more of the suppressor T-cell systems described by many laboratories (25–31). The role of Fc^+ T cells in active immunosuppression in these systems is at present under investigation. The data available at this time (unpublished observation) indicate that the Fc^+ T cells do not include the suppressor T cells responsible for the allotype-specific suppression described by the Herzenbergs (25). It has been demonstrated that concanavalin A (Con A) stimulation of T lymphocytes results in a nonspecific suppression of antibody responses, the suppression being mediated by a soluble factor (29, 31). The Fc^+ T cells described in the present report have been shown to respond very well to Con A whereas the Fc^- T cells respond very poorly or not at all to this mitogen.[2] Therefore, the Fc^+ T cells are likely candidates for the cells involved in this Con A-induced nonspecific suppressor mechanism.

Finally, it is, of course, possible that the Fc^+ T lymphocytes are involved in cell-mediated immunity. The increase in T cells bearing the Fc receptor as a result of allogeneic activation (7, 9) suggests that this may be the case. Indeed, the increase in Fc^+ T cells as a result of culture with KLH (8) might be explained by a cellular immune reaction to KLH. We are, therefore, currently investigating the ability of Fc^+ and Fc^- T lymphocytes to participate in cell-mediated immune reactions.

Summary

Utilizing the fluorescence-activated cell sorter (FACS) and washed murine antibody-antigen complexes formed in antibody excess, we have demonstrated the presence of the Fc receptor on the surface of a distinct subpopulation of

[2] Stout, R., and L. A. Herzenberg. The Fc receptor on thymus-derived lymphocytes. II. Mitogen responsiveness of T lymphocytes bearing the Fc receptor. Manuscript submitted for publication.

murine T lymphocytes. No differences in intensity of labeling with the complexes was observed when the Fc^+ T lymphocytes were compared with Fc^+ B lymphocytes. The majority of Fc^+ T lymphocytes are small lymphocytes as determined by light-scattering characteristics on the FACS. Separating Fc^+ from Fc^- T lymphocytes from spleens of mice primed 1 wk or 1 mo previously with keyhole limpet hemocyanin (KLH) revealed that the T cells capable of cooperating with DNP-KLH primed B cells to give an adoptive anti-DNP PFC response do not bear the Fc receptor.

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