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FUNCTIONAL STUDIES OF LYMPHOID CELLS DEFINED AND ISOLATED BY A FLUORESCENCE ACTIVATED CELL SORTER (FACS) FROM NORMAL AND ATHYMIC MICE[1]

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INTRODUCTION

Lymphocytes in the mouse have been separated into thymus dependent (T) and bone-marrow-derived (B) lymphocyte classes, each class expressing distinctive cell surface antigens and functions (1). Recent studies have suggested that a high degree of functional heterogeneity exists within the T and B lymphocyte classes. In addition to the maturational heterogeneity of the thymus cell population as a whole (2,3), "mature" T lymphocytes that participate in graft-vs-host and cytotoxic responses display functional heterogeneity with respect to homing properties and sensitivity to the cytotoxic effects of anti-lymphocyte and anti-Thy 1 antibodies (4).

Utilizing a quantitative fluorescence assay employing a fluorescence activated cell sorter (FACS), we have examined the differential expression on T lymphocytes of three cell surface markers--Brain Associated T(BAT) lymphocyte antigen, the Thymocyte-Bone Marrow (ThB) shared lymphocyte antigen (5), and the receptor for antigen-antibody complexes (Fc receptor) (6)--in an attempt to delineate T cell differentiation and maturation into functional subsets in normal and athymic mouse strains.

MATERIALS AND METHODS

Methods for the preparation of antisera, antigen-antibody complexes, cell suspensions, fluorescent staining procedures, analysis and separation of cells on nylon wool and the fluorescence activated cell sorter have been described in detail previously (2,4-7).

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Cell Transfer for Adoptive Anti-DNP Responses. The complete method has been published previously (8).

Lymphocyte Cytotoxicity Assay. EL-4 tumor cells were labeled with 150 μCi of ^{51}Cr per 10^8 cells (Amersham Searle, Arlington Heights, IL) for 30 min. at 37°C. The cells were washed and mixed with the sensitized lymphocytes at the effector-to-target ratios specified in each experiment. The cells were centrifuged at 200x g for 5 min. and incubated for 4 hr. at 37°C. The cells were then centrifuged at 500x g for 10 min. and aliquots of the supernatant counted in a gamma scintillation counter. Specific lysis was determined by the following equation:

$$\% \text{ Specific Release} = \frac{\text{cpm released} - \text{nonspecific release}}{\text{cpm released by 4x freeze thaw}} \cdot 100$$

Mixed Lymphocyte Reactions. An equal number (4×10^5) of responder lymphocytes and irradiated stimulator cells were cultured in 200 μl of RPMI 1640 supplemented to 5% with fetal calf serum and to 5×10^{-5} M with 2-mercapto ethanol. After 72 hr. the cultures were pulsed for 8 hr. with 1 μCi (^3H)-thymidine, harvested and counted.

Thymus Reticular Epithelial Cell (TRC) Monolayers. TRC Monolayers were prepared as previously described (9).

Fibroblast cultures were prepared by trypsinization of 12-14 day old mouse embryos and cultured in Eagle's medium supplemented to 10% with fetal calf serum.

RESULTS AND DISCUSSION

1. T lymphocyte differentiation in normal mice

Expression of ThB on Normal Thymocytes. Normal thymocytes may be separated on the FACS according to Thy 1 density into three major fractions as described by Fathman, et al. (2): large cells with a high Thy 1 density which are the rapidly dividing subcapsular thymocytes; small cells with an intermediate Thy 1 density; and medium size cells with a low Thy 1 density, which are the cortisone resistant immunologically competent medullary thymocytes (Table I). These subpopulations may be further fractionated on the basis of expression of a new membrane determinant, ThB, which is detected by antisera raised against intact MOPC104E plasmacytoma cells (5). As can be seen in Table I, ThB is present on all of

TABLE I

PROPERTIES OF THYMOCYTE POPULATIONS

Cell Size	Large	Small	Medium
% of Thymus	5 - 7	85 - 90	5 - 7
Anatomy	Subcapsular zone	Deeper cortex	Medulla
Life span	Rapidly turning over	Long-lived	Long-lived
Resistance to cortisone	No	No	Yes
Thy-1 (θ)	+++	++	+
TL	++	+	-
H-2	+	+	++
% ThB^+	94	56	3
% Fc^+	<1	10	9

the immature subcapsular thymocytes and on half of the small cortical thymocytes but is not present on the mature cortisone resistant medullary thymocytes. ThB is not present on peripheral T cells or on brain, liver, or kidney, but is present on peripheral B lymphocytes (5). The observation that ThB is present on splenic and lymph node B lymphocytes and immature cortical thymocytes but not on mature T cells suggests that ThB may be a determinant that is lost or masked upon T cell maturation. Our preliminary results indicate also that it is present in greater amounts on splenic B cells from pre-weaning or weanling mice than on 16-week and older mice. Thus it may be a B cell maturation marker as well.

Expression of the Fc Receptor on T Lymphocytes. Utilizing the FACS and murine antigen-antibody 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 distribution of Fc^+ T cells in the lymphoid tissue of BALB/c mice is summarized in Table II. An equal proportion (23%) of the T lymphocytes in lymph node and spleen bear the Fc receptor. A smaller proportion (<10%) of thymocytes bear the Fc receptor. These Fc^+ thymocytes appear in both the cortical and medullary regions of the thymus

TABLE II

FREQUENCY OF Fc^+ T LYMPHOCYTES IN BALB/cN LYMPHOID TISSUE

Tissue	T/Total	Fraction of Labeled Cells Fc^+/Total	Fc^+T/Total	Fc^+T/T
Spleen	.46 ± .08	.61 ± .10	.11 ± .04	.24
Lymph Node	.75 ± .09	.38 ± .08	.16 ± .06	.22
Thymus	.97 ± .01	.10 ± .04	0.1	0.1

(Table I) suggesting that the T cells acquire the Fc receptor relatively early in their maturational sequence.

Mitogen Responses. The responsiveness of FACS separated Fc^+ and Fc^- T cells to the T cell mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) was determined by culturing the Fc^+ and Fc^- fractions for 48-72 hr. with 2.5 μg/ml PHA or 1.5 μg/ml Con A, and pulsing for 8 hr. with 1 μCi (^{3}H)-thymidine prior to harvest. Both the Fc^- and Fc^+ T cell fractions responded to PHA. In contrast, the Fc^+ T cells, but not the Fc^- T cells, responded to Con A (Table III). Although all of the Con A responsive cells appeared in the Fc^+ fraction, the response of this fraction did not exceed the response of the unseparated splenic T cells. This inability to recover 100% of the Con A response on a per cell basis has been reported by other workers (10). It was hypothesized that, although only a small subset of T cells could respond directly to Con A, these responsive cells, upon Con A stimulation, might be capable of recruiting other T cells into mitosis. To examine this hypothesis, the Con A response of Fc^+ T cells mixed with either Fc^- T cells or with irradiated B cells, was examined. With irradiated B cells the response increased proportional to the number of Fc^+ T cells (Fig. 1). However, the response of any of the mixtures of Fc^- T cells and Fc^+ T cells was equal to the response of pure Fc^+ T cells, even though the Fc^- T cells alone responded poorly to Con A (Fig. 1). We concluded that the Fc^+ T cells, upon activation by Con A, were capable of recruiting Fc^- T cells into the mitotic response to this mitogen (11).

TABLE III

Con A AND PHA RESPONSIVENESS OF Fc^+ vs. Fc^- T CELLS

Splenic T Cells[a]	cpm ($\times 10^{-3}$) (^{3}H)-Thymidine/Culture[b] Medium Only	PHA	Con A
Unlabeled	0.15	138.5	49.3
Unseparated (AgAb)-labeled[c]	0.13	121.3	38.9
FACS-separated Fc^- [c]	0.16	124.3	3.4
FACS-separated Fc^+ [c]	0.18	80.5	34.5

[a] Splenic T cells were purified by nylon wool filtration. The purified T cell population contained <2% Ig^+ cells. Lymphocytes were incubated 30 min. at 37°C with or without preformed washed (AgAb) complexes, washed, and plated at a concentration of 10^6 cells/ml (2×10^5 cells/well) in the presence of 2.5 μg/ml PHA, 1.5 μg/ml Con A, or 25 μg/ml LPS. Cultures were harvested after 48 hr. of culture.

[b] Cultures were pulsed with 1 μCi ^{3}H-Thymidine 8 hr. before harvest. Numerical values represent average cpm of 3 cultures Std. Dev. omitted due to space limitations but were <20%

[c] After labeling with (AgAb) complexes, cells were incubated for 10 min. at 37°C in 0.1 mg/ml trypsin.

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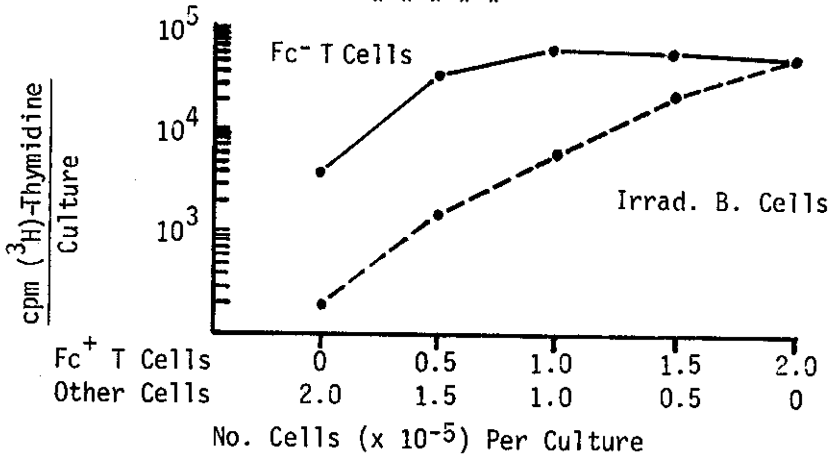

Fig. 1. Con A stimulation of Fc^+ T cells mixed with either Fc^- T cells or irradiated B cells (as filler cells). Fc^- cells recruited by Fc^+ cells into Con A response.

T-Cooperator Activity in Humoral Response. To assay cooperation in the adoptive secondary response to DNP-KLH, graded numbers of Fc^+ or Fc^- T cells isolated from KLH-primed mice were transferred into lethally irradiated syngeneic recipients along with 10 μg DNP-KLH and 5×10^6 anti-Thy 1 plus complement treated spleen cells from mice primed one month previously with 100 μg alum-precipitated DNP-KLH (DNP-primed splenic B cells). One week later, the recipient spleens were assayed for direct and indirect DNP-specfic PFC (8). As can be seen in Table IV, recipients of 5×10^6 DNP-primed B cells alone responded very poorly (<50 PFC/10^6 cells). This response increased linearly with increasing doses of KLH primed unseparated T cells. Recipients of 0.75×10^6 Fc^+ T cells did not mount a significant indirect DNP-PFC response. Since 23% of T cells were Fc^+ in this experiment, this number of Fc^+ T cells corresponds to 3×10^6 unseparated T. Thus the Fc^+ T cells have less than 4% of the helper activity of total T cells. Recipients of the Fc^- T cell fraction responded as well or better than recipients of unseparated T cells. Since all of the helper T cell activity was recovered on a per cell basis in the Fc^- T cell pool, T-coopertors are Fc^- T cells.

TABLE IV

Fc^- T CELLS ARE COOPERATORS IN DNP-KLH ADOPTIVE SECONDARY RESPONSE

B-Cell Source: DNP-KLH Primed Spleen		T-Cell Source		
Untreated	T Depleted Anti θ + C'	Nylon Passed Splenic T Cells		Indirect[b] PFC/10^6
10[a]		--		5,700
	5[a]	--		40
	"	Unseparated (77% Fc^-, 23% Fc^+)	3[a]	5,100
	"	"	1.5[a]	2,700
		Separated		
	"	Fc^-	1.5[a]	3,100
	"	"	0.75[a]	1,700
	"	Fc^+	0.75[a]	300

[a]Number of cells transferred x 10^6

[b]On day 7 after using TNP-burro red cells (8)

Cytotoxic T Cells. Fc^- and Fc^+ T cells isolated from spleens of mice sensitized 11 days earlier with EL-4 tumor cells were assayed for their ability to specifically lyse ^{51}Cr abeled EL-4 targets in vitro. Significant enrichment of cytotoxic activity was obtained in the Fc^+ T cell fraction (Fig. 2). At en effector-to-target ratio of 20:1, 36% specific lysis was obtained with unseparated T cells, 58% with Fc^+ T cells and less than 15% with Fc^- T cells (Fig. 2). It therefore appeared that cytotoxic effector T-lymphocytes bore the Fc receptor.

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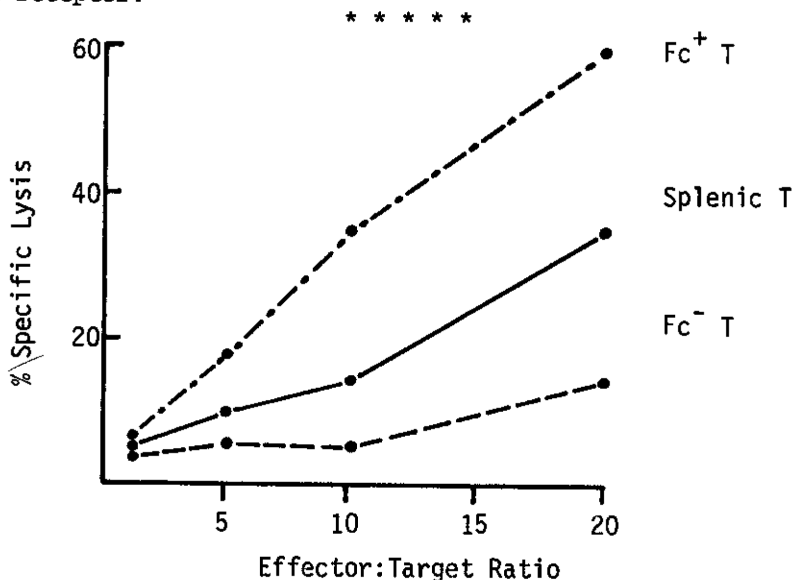

Fig. 2. Cytotoxic T Cells Are Fc^+. Mice injected i.p. with EL-4; 11 days later spleens were removed and separated into Fc^+ and Fc^-, then assayed for lysis (^{51}Cr release) of EL-4 targets.

* * * * *

Precursors of Cytotoxic T Cells. It has previously been suggested that the Fc receptor on T lymphocytes was acquired after allogeneic activation (12), implying that the precursors of cytotoxic lymphocytes were Fc^-. To examine this hypothesis Fc^- and Fc^+ T cells from normal mice were cultured with either syngeneic (BALB/c) or allogeneic (CSW) irradiated B cells. The ability to mount a proliferative response to the allogeneic cells was determined by pulsing the cultures on day 4 for 8 hr. with 1 µCi (^3H)-thymidine. Parallel cultures were assayed on day 5 for their ability to specifically lyse ^{51}Cr-labeled EL-4 target cells to determine which cultures

had contained the precursors of the cytotoxic effector cells. Both the Fc^+ and Fc^- T cell fractions mounted comparable proliferative responses to the allogeneic B cells. The Fc^+ T cell fraction also generated a substantial cytotoxic response (56% specific target lysis per culture) which was significantly higher than the response generated from unseparated T cells (39% specific target lysis per culture). However, in marked contrast to its proliferative response to the allogeneic stimulus, the Fc^- T cells generated very little cytotoxic activity (12% specific lysis per culture) in response to the allogeneic stimulus. Thus the precursors of cytotoxic lymphocytes, as well as the cytotoxic cells themselves, have the Fc receptor (9).

Quantitative fluorescence analysis (with the FACS) of Fc^+ and Fc^- cells cultured on syngeneic or allogeneic fibroblasts for from 0-5 days showed that all Fc^+ cells increase with time in ability to bind (AgAb) complexes. The increase was greater on allogeneic cells. Some (about 30%) Fc^- cells developed weak but detectable complex binding after 5 days culture on allogeneic cells. Only a few percent bound as much as similarly cultured Fc^+ cells and these could be due to multiplication of a few Fc^+ contaminants. Thus neither Fc^+ nor Fc^- cells develop into the other type upon culture on syngeneic or allogeneic fibroblasts.

Whether the cells which bind small amounts of complexes after culture of Fc^- T cells are another functional subset is of interest. These "low density Fc^+" T cells are neither cytotoxic cells nor helpers in a humoral response. They may include allotype suppressor T cells.

2. T cell presursors in congenitally athymic (nu/nu) mice.

Despite the absence of mature, immunocompetent T cells in nu/nu mice, precursors of T lymphocytes exist in these animals since bone marrow cells of nu/nu mice can repopulate the thymi of lethally irradiated normal recipients (14). Similarly, a thymus implanted into a nu/nu mouse repopulated by cells of host (nu/nu) origin (14). It therefore appears that these mice have no defect in either the lymphocyte stem cell population or in development of pre-T cells (prothymocytes).

In this study, we have used a rabbit antibody, raised against mouse brain (15) and rendered it T specific by absorption, to detect and separate T-precursor cells from

nu/nu spleen. To distinguish the T antigen detected by this antiserum from the allo antigen Thy 1 (θ), we have designated it Brain Associated T-lymphocyte (BAT) antigen.

The fluorescence and scatter (size) profiles of nu/nu spleen cells (Fig. 3) show a substantial fraction of cells with BAT staining and of large cells. Fluorescence gated scatter analysis shows that most of the BAT^+ cells are large. Stained smears of FACS isolated BAT^+ cells have a high proportion of large highly basophilic lymphoblasts. The BAT^+ cells in BALB/c are nearly all small lymphocytes. Nu/nu spleen and lymph node have proportions of BAT^+ cells approaching those of their congenic normal BALB/c counterparts (spleen 32-43% and lymph node 26-74%, respectively, for nu/nu and BALB/c). The intensity of staining of BAT^+ cells is not notably different (certainly not less) in nu/nu than in normal. On the other hand Thy-1 staining of nu/nu spleen and lymph node is much weaker (4, unpublished observation).

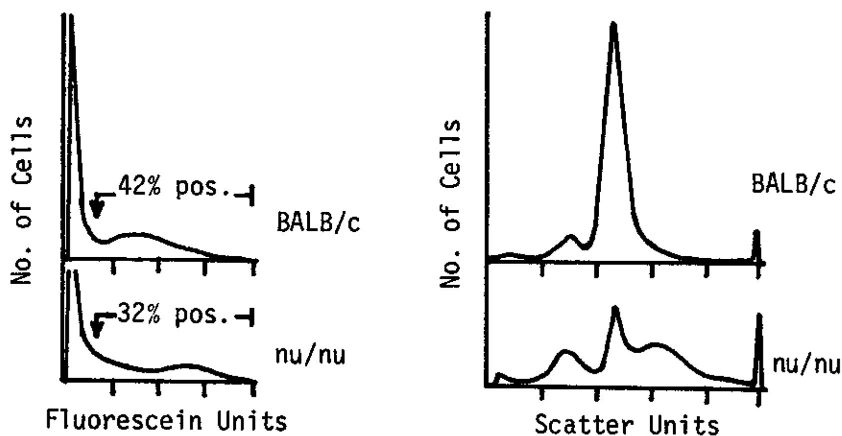

Fig. 3. FACS analysis of BALB/c and nu/nu Spleen Cells Stained for BAT.

It has previously been demonstrated that responsiveness of nu/nu splenic lymphocytes to Con A can be increased by culturing the spleen cells on monolayers of thymic reticular epithelial cell (TRC) monolayers (9). To determine whether the BAT positive or negative cells from nu/nu spleen contained the precursor cells affected by the TRC monolayers, unseparated spleen or FACS-separated BAT^+ or BAT^- spleen cells from nu/nu mice were cultured on syngeneic (BALB/c) fibroblasts or TRC (BALB/c) monolayers for 24 hours, washed, and assayed for

their ability to respond to PHA, Con A, or LPS. Cultivation of unseparated nu/nu spleen cells on TRC monolayers resulted in a significant increase in Con A responsiveness (Table V). Similarly, culture of BAT+ nu/nu spleen cells on TRC monolayers resulted in an increase in Con A responsiveness. In contrast to the BAT^+ cells, the BAT^- cells exhibited no responsiveness to Con A either before or after cultivation on TRC monolayers (Table V).

These studies in nu/nu mice indicate that committed pre-T cells, or prothymocytes, exist in the peripheral lymphoid organs of nu/nu mice, bear a surface marker (BAT) characteristic of T lymphocytes, and are responsive to the inductive influence of TRC. These cells correspond to the prothymocytes defined by Boyse and coworkers (16).

TABLE V

MITOGEN RESPONSE OF nu/nu SPLEEN CELLS BEFORE AND AFTER INDUCTIVE CULTURE

	Before					After			
	Control	Con A	PHA	LPS		Control	Con A	PHA	LPS
Unsep.	.61*	.70	.35	6.5	Fib†	.35	.42	.51	5.3
					TRC^Ψ	.53	1.2	.83	7.6
BAT^{+}§	.72	3.7	.67	10.5	Fib	.72	3.7	.67	10.5
					TRC	1.2	12.7	2.7	14.0
BAT^-	.14	.35	.23	38.	Fib	.49	.46	.72	21.1
					TRC	.65	.92	.60	18.2

* (^{3}H)-Thymidine cpm x 10^{-3}

† Mouse embryo fibroblasts

Ψ Thymic reticular epithelial cells

§ Brain associated T-antigen

SUMMARY AND CONCLUSIONS

We can now sketch a picture of T lymphocyte differentiation in the mouse based upon these and other studies. Hematopoietic stem cells which first arise in yolk sac and then appear in fetal liver and adult bone marrow and spleen give rise to committed T cells or prothymocytes. These are large lymphoid cells which bear a T surface antigen, BAT, and which in normal mice go to the thymic cortex where they continue maturation and differentiation presumably under the influence of thymopoietin (16) secreted by the thymic epithelium.

In the athymic mouse, BAT^+ cells increase markedly in number in all lymphoid tissues although very few Thy-1 (θ) positive and functional T cells arise. The increase in BAT^+ cells in the bone-marrow may be due to the absence of feedback regulation by either a thymus or by mature T cells.

The ThB antigen marks immature thymocytes and is lost as the subcapsular cortical cells migrate towards the medulla. ThB may also mark prothymocytes since all nu/nu lymphoid tissues have large numbers of ThB large cells. Whether BAT and ThB are on all the same cells is under current study.

Thymic reticular epithelial cells (TRC), like thymopoietin, induce prothymocytes to develop Con A and MLR responsiveness. They may do this via increasing cAMP in the target cells (16).

At least two and probably at least four separate lineages of T cells have been found, each with its own functional activity. The Ly-1 and Ly-2,3 antigens (17,18) and the presence or absence of Fc receptors (11,13) mark the four T cell subsets. These become functionally mature as differentiation proceeds in the thymus and in the periphery (spleen and perhaps lymph nodes as well). Antigen is apparently not involved in this differentiation.

The overlap of the Fc^+ and Ly subsets is now being studied but it is already clear that they do not coincide but define four cell types between them. Some Ly-1 and Fc^- cells are cooperators in humoral and cellular immune reactions and proliferate in an MLR. Some Ly-2,3 and Fc^+ cells are precursors of cytotoxic cells and effectors. T suppressors (19,20) may fall into both categories. Cells appear to change neither Ly-1,2,3 type nor Fc receptor type during antigen independent nor dependent differentiation.

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DISCUSSION

Sprent, London: You showed that some Fc-receptor negative T cells acquired the Fc-receptor by culturing on allogeneic fibroblasts. Presumably, the fibroblasts would not present MLR determinants. Do you find the same thing if your culture the cells with e.g. spleen cells?

Herzenberg: It is very difficult to do this with spleen cells, because one has to distinguish between responding and stimulating cells. That is why fibroblasts were used.

Krammer, Basel: We have tried to make activated T cells using an

allogeneic system, by infusing parental cells into irradiated F1 animals. From the spleens of such animals we recovered a population of pure T cells which contains about 50% EA-rosette forming cells. From these, essentially pure Fc-receptor positive cells, as well as Fc-receptor negative cells, were isolated by velocity sedimentation. Both populations are effective killer cells. Together with John Sprent and Leslie Hudson we looked at T-TDL, which are also activated T cells; these T-TDL are all Fc-receptor negative; they are also effective killer cells. The second point is that when you take these Fc-receptor negative T-TDL and infuse them into irradiated F1 hosts, the number of Fc-receptor positive cells in the spleens of these hosts is twice as high as in animals which had just been irradiated. What this would suggest is that these Fc receptor negative T-TDL become Fc-receptor positive upon re-infusion into new allogeneic hosts.

Herzenberg: As you stated, differences in methodology may be crucial. You are using another assay, EA-rosette formation. It may be worthwhile to look into the differences of course; for a long time it has been stated that Fc-receptors were only on B cells; then on activated T cells. Now I think many other people have also found them on non-activated T cells. For instance Henkart showed that you can bind T cells to antigen-antibody complexes of yet a different sort. Why EA-rosette formation is apparently not a good method of finding Fc-positive T cells, I don't know.

Krammer: One further comment. The T-TDL, which as I said are activated T cells, have been checked with a variety of methods, none of which showed any expression of Fc-receptors on those cells, even though they are killer cells.

Herzenberg: In general, binding to T cells is not as easy as to B cells in many situations. Not just for EA; even staining T cells with anti-H2 in the cold is not easy as staining B cells. In many situations activated T cells are easier to stain than non-activated T cells. I can only say this tells us something about the membrane of these cells.

Fridman, Paris: Have you looked whether your Fc-receptor positive killer T cells could also be cytotoxic in an antibody-dependent assay? Could part of your killing not be explained by a K-cell mechanism?

Herzenberg: Actually Bob Stout has not yet done that. I don't think that the killing is at all likely to be due to an antibody-dependent

mechanism, because first of all the killing is certainly due to T cells, sensitive to anti-Θ plus complement. Furthermore, during stimulation on fibroblasts you clearly get a proliferation of the cells that are involved. I don't think the K cells would have proliferative capacity. This is clearly something which should be tested directly.

G. Möller, Stockholm: I'm glad that the so-called T cells in nude mice have no function and that the only thing they are distinguished by is the marker. Isn't it possible that the marker on such cells is for entry into the thymus, where they become T cells?

Herzenberg: I think you are getting into semantics. I said they were pro-thymocytes, which is exactly what you are saying, and what Boyse, for example, would call them. These cells share an antigen with functional T cells. They can be turned into functional T cells by appropriate inducing agents, which is probably happening in the thymus. The intensity of staining with the T-specific and brain antibody is the same for pre-thymocytes, thymocytes and mature T cells. I think the basic antigenic protein macromolecule is the same in all these cells, but some determinants are added on or decreased during differentiation. For instance, you find more Thy-1 on primitive thymocytes than on mature cells.

G. Möller: How does your detection sensitivity with a fluorescence-activated cell sorter compare to a fluorescence microscope? I don't think, for instance, that in a microscope anyone has seen 30% of T positive cells in nude mice.

Herzenberg: I you have a good fluorescence microscope you can in fact see most of what you can see on the cell sorter. It takes longer, you can look at 100 cells in a couple of hours as opposed to 10,000 cells in a minute, but the sensitivity can be the same. It is harder to quantitate by eye, since the eye is much more sensitive to differences in intensity than it is to absolute intensity, so if you have patching or capping the cells look brighter and you can pick them up more easily than if they are diffusely stained. You should use a photometer if you want quantitation.
As to your last point, Roelants and Loor have also reported T positive cells in nude mice, using a very similar antiserum.

Rubin, Copenhagen: I want to take up the point about K cell activity among the ATC's - the Fc-receptor positive cells do work in the anti-

body-dependent cell-mediated cytotoxicity system. However, these cells seem to have very little Θ on their surface, at least in our hands they are Θ-negative. Another point concerns the development of Fc-receptor positive cells by injecting thymocytes into irradiated hosts; we get as many EA-rosetting T cells if we inject thymocytes into syngeneic irradiated recipients as we do in semi-allogeneic recipients. Furthermore, with the serum from the syngeneic injected thymocyte recipients, you can induce EA-rosette formation in normal thymocytes.

Herzenberg: When we cultured our Fc-receptor negative T cells on syngeneic fibroblasts, they did become positively staining with immune complexes, although much weaker than when you start with Fc-receptor positive cells. All depends of course on the assay system, and the definition of positive. If you use large aggregates it is quite difficult to distinguish between positive and negative. You can get 100% of thymocytes stained if you set you threshold at a different level, which is fairly arbitrary.