PHENOMENON OF HUMAN T CELLS ROSETTING
WITH SHEEP ERYTHROCYTES ANALYZED
WITH MONOCLONAL ANTIBODIES

"Modulation" of a Partially Hidden
Epitope Determining the
Conditions of Interaction between T Cells
and Erythrocytes*

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Human T cells are characterized by their ability to form spontaneous rosettes with sheep erythrocytes (E)[1] (1–5). This property is currently used to enumerate T cells because it is restricted to these cells. Yet it is still poorly understood. It appears to be a complex phenomenon, depending exquisitely on certain requisites of the procedure used, for example, temperature and time of cells' coincubations and pretreatment of E with various chemical compounds, so that the T lymphocyte population marked by this phenomenon will critically depend upon the conditions followed during the assay used to prove it. For instance, pretreatment of E with neuraminidase or 2-amino-ethylisothiouronium bromide (AET) will permit the detection of the largest population of T cells, whereas interaction with E at 37°C will permit only the detection of thymocytes, or T lymphoblasts (5–12).

Recently (13), a monoclonal antibody (MAb) termed 9.6 was obtained, which has permitted the identification of a T lymphocyte surface molecule necessary to permit their interaction with E. We have obtained another MAb, termed anti-D66 (14), which identifies an epitope that also appears, as demonstrated here, to be involved in E-rosette formation. By contrast to 9.6, anti-D66 does not block rosettes when E have been pretreated with neuraminidase or AET, although it blocks E-rosettes made both at 4°C and 37°C. Thus, anti-D66 is extremely useful for investigating the phenomenon of E-rosette formation.

[1] *Abbreviations used in this paper:* AET, 2-amino-ethylisothiouronium bromide; ALL, acute lymphoblastic leukemia; BFUE, erythroid burst-forming unit cells; CFUC, colony-forming unit cells; CLL, chronic lymphocytic leukemia; Con A, concanavalin A; E, erythrocytes; E⁺-PBL, peripheral blood lymphocytes forming E-rosettes; Fab-MIg, Fab fragments of immunoglobulin anti-mouse immunoglobulins; GMIg, goat immunoglobulin anti-mouse immunoglobulins; LL, lymphoblastic lymphoma; MAb, monoclonal antibody; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; SACI, *Staphylococcus aureus* Cowan I; Séz, Sézary.

We demonstrate here that, on the thymocyte surface, D66 and 9.6 epitopes are carried by the same molecule; we also show that, although 9.6 epitopes are readily exposed on the cell surface, a large fraction of the D66 epitopes is covered by the glycocalixte from which it can be unmasked by anti-D66 sensitization plus anti-mouse immunoglobulins (Ig). Our observations offer a view on the respective roles of 9.6 and D66 epitopes in determining the conditions of interactions between T cells and E. Extending these observations to malignant T cells populations, this view would account for the "aberrations" observed in the E-rosette-forming properties of these populations (14, 15).

Materials and Methods

Preparation and Characterization of Monoclonal Antibodies. Mice from the high responder strain selected by Dr. G. Biozzi (16) were immunized with a single intravenous injection of 5×10^7 thymocytes collected from a child undergoing cardiac surgery. 4 d later, spleen cells were fused with cells from the MOPC 21 NS1/1 plasmocytoma cell line, as previously described (16). The supernatants were screened on a panel of various lymphoid cells using a C'-dependant microcytotoxicity assay (17). Hybrids were serially cloned four times using a limiting dilution method. Class and subclass of the antibody were assessed by immunodiffusion with rabbit antisera (Litton Bionetics Inc., Kensington, MD) and proved to be an IgG_{2b} molecule. Hybrid cells were innoculated intraperitoneally into nude mice raised under sterile conditions. Ig were concentrated from the ascites by ammonium sulphate precipitation (40% saturation at 0°C), followed by washing, and desalted either by dialysis or by chromatography (ACA-202; IBF-Pharmindustrie, Villeneuve-La-Garenne, France). The amount of antibody was measured by immunodiffusion and compared with the total protein content, as assessed by the Folin-Ciocalteu method. Next, biotin was fixed to Ig according to the general procedure described in ref. 18. The Ig solution was adjusted to 1 mg/ml in 0.2 M, pH 8.8 bicarbonate buffer, containing 0.15 M sodium chloride. 2 mg of N-hydroxysuccinimide biotinate (Act-Biotine; IBF-Pharmindustrie) was dissolved in 0.5 ml of anhydrous N-methylformamide. 200 μl of Act-Biotine was stirred into this solution and maintained for 15 min at 20°C. Next, 100 μl of 0.1 M, pH 6.0 ammonium chloride was added, and the preparation was desalted by chromatography.

Preparation and characterization of 9.6 antibody used in the present investigation have been described by Malek et al. (13). Preparation and characterization of rabbit anti-human T cell antisera were extensively described elsewhere (19).

Cell Preparations. Mononuclear cells were obtained from peripheral blood (PBL) by Ficoll-Hypaque density centrifugation. Cells forming rosettes with sheep erythrocytes (E^+-PBL) were isolated after removing plastic adherent and iron particles phagocytosing cells by rosetting with E_{AET} (see below) and centrifuging on Ficoll-Hypaque. E^- cell fraction was taken as the supernatant cell fraction after two sequential Ficoll-Hypaque centrifugations of PBL allowed formation of rosettes with E_{AET}.

Thymocytes were obtained from children undergoing cardiac surgery, from bone marrow normal healthy volunteers, for the purposes of allogenic bone marrow grafts, and from lymph nodes and spleens from cadaveric kidney donors. Cells were dispersed and prepared as described above.

Malignant lymphoid cells were obtained from peripheral blood, lymph nodes, or pleural effusions. Specimens retained for investigation contained >85% malignant cells, as assessed by morphological criteria (20). Procedures for cell prepations and cryopreservations have been described elsewhere (21).

Serological Analysis. Reactivity of anti-D66 MAb with cells was assessed using a complement-dependent microcytotoxicity assay. It was also assessed by microfluorometry using a cytofluorograf (system 30 L; Ortho Instruments Co., Westwood, MA). Unless otherwise stated, cells were incubated for 10 min at room temperature with an excess amount of anti-D66 preparation in phosphate-buffered saline (PBS), containing 0.2% sodium azide. The second reagent used was a fluorescinated goat $F(ab')_2$ anti-mouse IgG (Tago Inc., Burlingame, CA), tested for its reactivity with IgG_{2b}.

Alternatively, a proper dilution of biotinylated anti-D66 preparation in 0.2% PBS-azide was incubated with cells at room temperature for 15 min. After washing the cells, excess amounts of fluorescinated-avidin (IBF-Pharmindustrie) were added, and cells were incubated for 20 min at room temperature followed by three washes in PBS-azide.

In each experiment, controls included the same cell preparation sensitized with an irrelevant IgG$_{2b}$ MAb, followed by incubation with the second reagent.

E-rosette Formation.   E were collected weekly and kept in Alselver solution. After extensive washings with PBS, E were treated with AET (Sigma Chemical Co., St. Louis, MO) or neuraminidase (Behringwerke AG, Marburg, West Germany), as previously described (8, 10).

The detailed procedures for E-rosette formation at 4°C, 37°C, and "active E-rosette tests" were described in detail elsewhere (2, 22, 23). In blocking experiments, cells were incubated with the appropriate dilution of MAb, washed twice in PBS-containing bovine serum albumin, and assessed for rosette formation. Numerous checks were made using irrelevant MAb of the same subclass reacting with the lymphocyte surface; these did not induce rosette inhibition.

Isolation and Characterization of Cell Surface Molecules.   Cells from HDMAR or KE37 lines were labeled by overnight incubation with [^{35}S]methionine (CEA, Gif sur Yvette, France) (1 mCi of [^{35}S]methionine by 5 × 10^7 cells incubated in 10 ml of methionine-free medium). Labeled cells were lysed by incubation for 45 min at 0°C with 0.5% Nonidet P40 in 10 mM Tris HCl, pH 8.0, 10 mM iodoacetamide, 2 mM phenylmethylsulfonyl chloride buffer. The lysates were centrifuged at 10,000 g for 3 min before storage at −70°C and recentrifuged upon thawing. Before use in immune precipitation analysis, the lysates were sequentially allowed to react with normal rabbit sera and fixed *Staphylococcus aureus* Cowan I (SACI) and next with rabbit anti-mouse IgG$_{2b}$ and SACI. Immunoprecipitations were performed by coincubations of cell lysates with D66 or 9.6 for 30 min, followed by addition of SACI previously reacted with saturating amounts of R-MIg$_{2b}$. After 15 min, the immune complexes were extensively washed in NaCl, EDTA, and Tris buffers and dissociated as described elsewhere (47). Finally, supernatants were analyzed on a 30-cm long, 7.5–15% gradient of acrylamide gel containing 0.1% SDS. Fluorography was carried out as described (48), using Kodak RPX-O-mat films (Eastman Kodak Co., Rochester, NY) at −70°C.

Results

The Cellular Distribution of D66 Epitope among Leukocytes.   Fig. 1 shows the density of D66 antigen on T cell populations, as measured by flow-cytometry. Thymocytes, peripheral blood T cells (E$^+$-PBL), and T lymphoblasts all gave a single peak on the fluorescence histogram, but it is clear that the density of D66 varies among these populations; it was high on thymocytes and stimulated T cells, particularly with
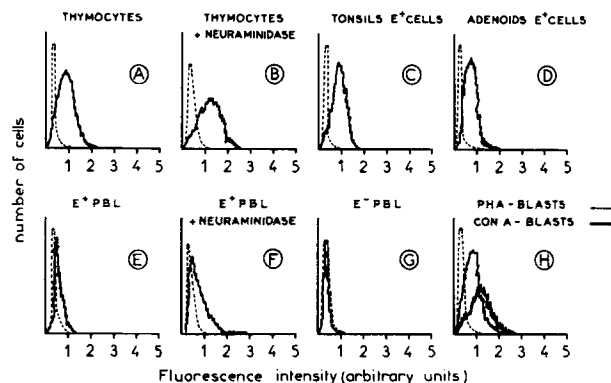

FIG. 1.   Fluorescence histograms obtained with various normal T cells populations labeled with anti-D66 plus fluorescinated goat anti-mouse Ig. Similar results were obtained using a biotine-avidin system.

TABLE I

D66 Distribution among Leukocytes

Cell populations	C'-dependent* cytotoxicity assay	Immunofluorescence assay‡	
		Percent of recognized cells	Intensity§
Thymocytes	100	100	+++
E⁺-PBL	100	100	+
E⁻-PBL	0	0	0
Granulocytes	0	NT‖	NT
E⁻ bone marrow cells	0	0	0
E⁺ spleen cells	100	NT	NT
E⁻ spleen cells	0	NT	NT
E⁺ lymph nodes cells	100	NT	NT
E⁻ lymph nodes cells	0	NT	NT
E⁺ tonsil cells	40.60	100	++
E⁻ tonsil cells	0	0	0
E⁺ adenoid cells	40.60	100	++
E⁻ adenoid cells	0	0	0
B CLL cells¶	0/5	0/5	0
B cell lymphomas¶	0/6	0/6	0
Non-T lymphoid cell lines¶**	0/8	0/8	0
Non-T non-B ALL¶	0/15	0/15	0
Acute myeloblastic leukemia¶	0/5	0/15	0

* Percent of destroyed cells.
‡ Performed by flow cytometry as described in Materials and Methods.
§ See also Fig. 1.
‖ Not tested.
¶ Number of positive cases per number of tested cases.
** The following cell lines were investigated: Daudi, Raji, 8866, Ramos, NALM 6, NALM 16, K562, and Reh 6.

concanavalin A (Con A) and very low on E⁺-PBL. It is worth noting that, as shown in Fig. 1, the density of D66 on thymocytes and, to a lesser extent, on E⁺-PBL, greatly increased after these cells were treated with neuraminidase. The mean density of D66 on the surface of E⁺ cells from tonsils and adenoids appears to be closer to the values observed with thymocytes or stimulated T cells than of peripheral blood T cells.

Anti-D66 is strongly cytotoxic with complement. In Table I, it can be seen that anti-D66 plus complement destroys all the cell populations displaying D66 in flow cytometry, including populations with very low density. This is also true for malignant T cell populations (Table V). However, tonsil and adenoid T cells appeared difficult to destroy; when we treated PBL with anti-D66 plus complement, we no longer observed a mitogenic response to phytohemagglutinin (PHA), Con A, or allogeneic cells, nor could we observe the generation of specific cytotoxic T cells. At the effector phase, these cells were also eliminated by anti-D66 plus complement (results not shown).

In the bone marrow, a minor subpopulation appeared to display D66 antigens,

which disappeared after removal of E-RFC or killing cells with hetero-anti-T cell antiserum plus complement (see Table I). To assess whether D66 would be carried by precursor hematopoietic cells, we studied whether colony-forming unit cells and erythroid burst-forming unit cells would be eliminated by anti-D66 plus complement. No effect was observed on the number of growing colonies after such a treatment (results not shown). D66 antigen was not detectable on normal B cells from various organs as well as monocytes and granulocytes, nor was it detectable on B cell lines or on the cells from the various myeloid, non-T non-B, and B lymphoid malignancies we studied (see Table I). Thus, D66 epitope, among leukocytes, appears to be restricted to T cells. Moreover, the D66 molecule is actually synthetized by T cells because destruction of D66 with pronase was followed by in vitro resynthesis. We performed this experiment both with normal T cells and with a T cell line (results not shown).

Blocking E-Rosette Formation with Anti-D66. As shown in Fig. 2, we observed that precoating T lymphocytes with anti-D66 inhibited E-rosette formation both at 4°C, 37°C, and after the procedure of active E-rosettes (2, 23) in a dose-dependent manner until completely inhibited. However, we observed that anti-D66 had no blocking effect when E were pretreated with AET or neuraminidase. We observed these blocking patterns of anti-D66 on E-rosette formation of E$^+$-PBL, thymocytes, and mitogen-stimulated lymphoblasts. Interestingly, when we stimulated T cells with mitogens, their D66 surface density increased simultaneously with their ability to form stable E-rosettes at 37°C (Table II).

We next investigated whether the D66 epitope would be directly involved in the interactions between E and T cells by capping experiments. As anti-D66 alone did not induce capping, we added a goat anti-mouse Ig (GMIg). Table III shows that after we induced an almost complete loss of D66 from the T cell surface, they were no longer able to form rosettes with E, both at 4°C and 37°C. This could be achieved, however, according to the experiment, after at least three successive steps of capping procedure (i.e., successive addition of anti-D66, GMIg and incubation), when we observed a significant decrease in D66 density on the cell surface in flow-cytometry, and it took six successive cycles before D66 density would reach very low values (Fig. 3, E and F). In a parallel manner, the ability of T cells to form E-rosettes at 4°C and 37°C decreased with successive capping (Table III).

Unmasking Hidden D66. Surprisingly, after a single cycle of capping with GMIg, and despite the observation of a bright fluorescent cap, rosette inhibition produced
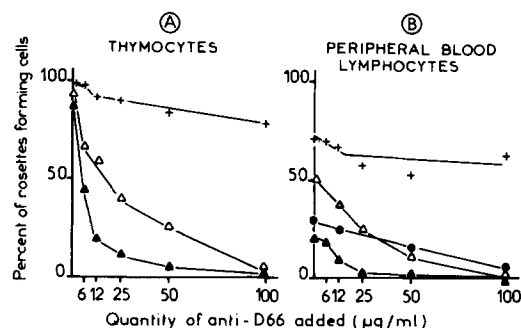

Fig. 2. Inhibition of E-rosette formation by increasing amounts of anti-D66. (+) E$_{AET}$ rosettes; (△) E-rosettes at 4°C; (▲) E-rosettes at 37°C; and (●) "active" E-rosettes.

TABLE II

Kinetics of Variations in D66 Densities on the Surface of T Cells after Mitogenic Stimulation

Hours	D66 surface density*	Percent E.RFC at 37°C§
PHA‡		
0	+	30 ± 9
12	++	60 ± 10
24	+++	76 ± 7
48	+++	85 ± 7
72	+++	87 ± 6
Con A‡		
0	+	30 ± 10
12	+++	90 ± 10
24	++++	95 ± 7
48	++++	92 ± 6
72	++++	92 ± 5
	++++	

* Measured by flow fluorometry; +, density analogous to $E^{\pm}$-PBL; +++, density analogous to thymocytes (see Fig. 1).

‡ 10^5 cells were cultivated in 200 μl of RPMI-FCS containing the optimum dilution of PHA (Wellcome Laboratoires, Beckenham, England) or Con A (Sigma Chemical Co., St. Louis, MO.)

§ Rosette-forming cells

by anti-D66 was completely raised (Table III and Fig. 4). To further investigate this phenomenon, we first used an Fab fragment of a rabbit anti-mouse Ig (Fab-MIg). Again, as shown in Table III, with a single addition of Fab-MIg on anti-D66-sensitized T cells, rosette inhibition was raised. Control tests done in fluorescence assay showed that the amount of Fab-MIg added could totally inhibit subsequent binding of fluorescent GMIg (FIg. 3 B). Yet, on cells sensitized with anti-D66 plus Fab-MIg, subsequent incubation with anti-D66 revealed that a large number of D66 epitopes could then be detected on the whole cell surface (Fig. 3 C). Thus, binding of an Fab fragment or of whole molecules of anti-mouse Ig to anti-D66 fixed to the cells induced the appearance of large amounts of D66 epitopes not readily accessible on the cell surface. Most probably these additional epitopes allowed T cells to reform E-rosettes.

Such a phenomenon—unmasking D66 not readily accessible on the cell surface, induced by fixation of GMIg or Fab-MIg on anti-D66—could not be blocked, as shown in Table III and Fig. 3 C′, by cytotalasin B up to a concentration of 100 g/ml, by incubation at 4°C, or in sodium azide (0.2%) alone. It was only by maintaining the T cells both at 4°C and in the presence of sodium azide, after sensitization with anti-D66, that further addition of anti-MIg no longer raised the blocking the E-rosette formation, nor induced unmasking of D66 as revealed by flow cytometry (Fig. 3 C″).

Since observing that neuraminidase unmasked large quantities of D66 on the thymocyte surface, we wondered whether the effects of anti-MIg on anti-D66-sensitized thymocytes would be raised or reduced after pretreatment with neuraminidase. From Table III, it can be seen that either GMIg or Fab-MIg had a reduced effect on the inhibition of E-rosette formation induced by anti-D66 on neuraminidase-pretreated thymocytes; from Fig. 3 G, it can be seen that the fluorescence intensity of

TABLE III

*Anti-D66, Anti-Mouse Ig, Fab Anti-Mouse Ig, and the Inhibition of E-Rosette Formation of Neuraminidase-treated or Nontreated Thymocytes**

Cell sensitization	Untreated thymocytes			Neuraminidase-treated thymocytes		
	E 37°C	E 4°C	E_{AET}	E 37°C	E 4°C	E_{AET}
Medium	93	96	95	96	98	95
Anti-D66	1	12	95	5	18	96
Anti-D66 + GMIg	87	89	NT‡	35	45	NT
Anti-D66 + Fab-MIg	92	98	NT	27	39	NT
With cytochalasin B						
Anti-D66 + GMIg	89	90	NT	NT	NT	NT
Anti-D66 + Fab-MIg	88	86	NT	NT	NT	NT
At 4°C + PBS-Az						
Anti-D66 + GMIg	NT	63	NT	NT	16	NT
Anti-D66 + Fab-MIg	NT	56	NT	NT	18	NT
Successive sensitizations						
(Anti-D66 + GMIg) × 3	51	76	NT	NT	NT	NT
(Anti-D66 + Fab-MIg) × 3	48	62	NT	NT	NT	NT
(Anti-D66 + GMIg) × 6	24	51	85	12	35	95
(Anti-D66 + Fab-MIg) × 6	18	48	92	7	42	91
(Medium + GMIg) × 6	92	87	89	95	87	86

* Results expressed as percent of E-rosette-forming cells.
‡ Not tested.

anti-D66-labeled cells was readily maximal, and further addition of anti-D66 did not result in an increase in fluorescence.

The Relationship of D-66 Epitopes with 9.6 Epitopes. 9.6 is an MAb obtained by Kamoun et al. (13), which has been shown to block E-rosette formation. In addition, the 9.6 epitope was shown to be involved in the interactions between E and T cells by lysostripping experiments (13). We have thus investigated the relationship between the epitopes defined by 9.6 and anti-D66. Several major differences immediately became apparent: (a) 9.6 could block rosette formation with E pretreated with AET as effectively as it could block rosette formation with nontreated E; (b) on a weight for weight basis, 9.6 was much more efficient in blocking E-rosette formation than was D66; and (c) in contrast with D66, the density of 9.6 on E^+-PBL is similar to its density on thymocytes (results not shown).

Second, we looked to see whether 9.6 could block subsequent fixation of anti-D66 and vice versa. Fig. 5 clearly shows that no cross-blocking occurred between the fixation of anti-D66 and 9.6 to thymocytes, demonstrating that they react with two different epitopes.

Third, are D66 and 9.6 epitopes linked on the cell surface? We investigated this point in a series of cocapping and lysostripping experiments. The results are displayed in Table IV. It is clear that when we used 9.6 as the first antibody to induce cap formation on thymocytes, the capped cells could no longer be restained or destroyed by anti-D66. When we used anti-D66 as the first antibody to induce cap formation,
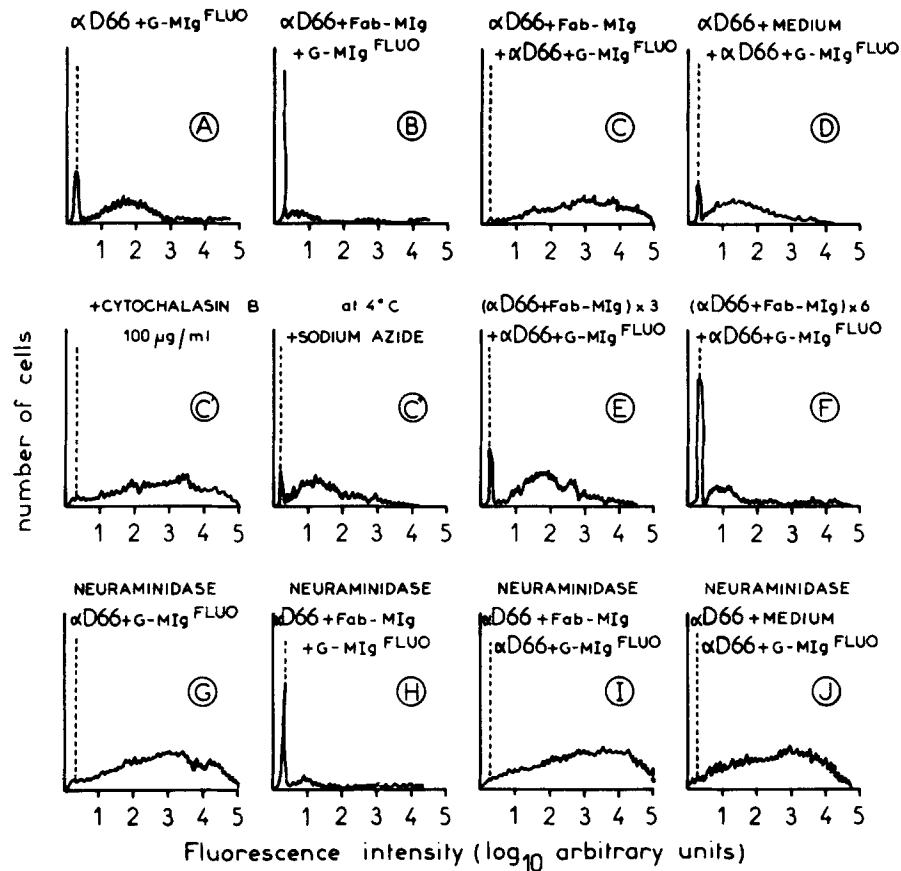
Fig. 3.   Fluorescence histograms obtained after various procedures of thymocytes sensitization with whole anti-mouse Ig or a Fab fragment.

most of the cells were restained by anti-D66 and 9.6. However, their susceptibility to lysis by anti-D66 as well as 9.6 was greatly decreased, whereas little effect was observed on their ability to be killed by an irrelevant MAb.

Fourth, are D66 and 9.6 carried by the same molecule? We demonstrated this by sequential radioimmunoprecipitations, using radiolabeled T cell lines. From Fig. 6, it is clear that both 9.6 and anti-D66 precipitated a single polypeptide of $\approx 50,000$ mol wt. In addition, sequential precipitations with anti-D66 followed by 9.6 or with 9.6 followed by anti-D-66 showed that the same polypeptide is indeed recognized by both antibodies.

Correlations between 9.6 and D66 Surface Densities of T Lymphoblastoid Cells and Their Abilities to Form E-Rosettes.   The correlation between D66 surface densities of normal T cells and their ability to form stable E-rosettes at 37°C led us to investigate T lymphoblastoid cells either in long-term culture or from specimens freshly collected from patients. All the lymphoblastoid cell populations we investigated were differentiated within the T cell axis because they reacted with several anti-T cell antisera (19) and MAb-defining epitopes considered to be T cell specifics and lacking markers found on B or non-T non-B lymphoid cells. The results were clear-cut: 9.6^-, $D66^-$ cell
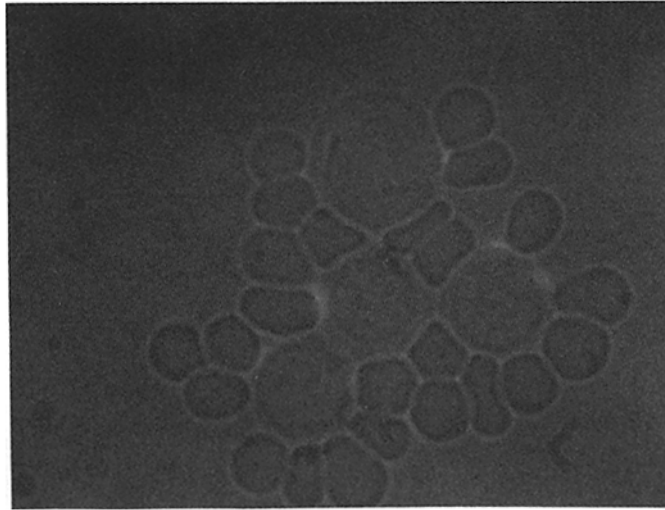
FIG. 4. Rosette formation at 37°C between untreated E and thymocytes sensitized with anti-D66 followed by fluorescinated anti-mouse Ig and allowed to cap. The amount of anti-D66 used would totally inhibit E-rosette formation when used alone.
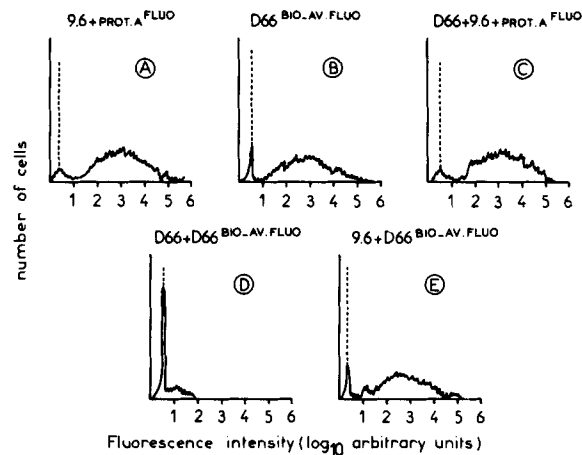

FIG. 5. Fluorescence histograms obtained after labeling thymocytes with 9.6. and fluorescinated protein A (anti-D66 do not fix protein A) and/or biotinylated anti-D66 plus fluorescinated avidin.

populations did not form E-rosettes, either with E_{AET} or with non-treated E. Only cell populations displaying high D66 surface densities formed E-rosettes stable at 37°C. 9.6^+, low surface density D66 lymphoblastoid cells formed significant amounts of E-rosettes stable at 4°C; finally, 9.6^+, $D66^-$ cells only formed rosettes with E_{AET}. It is notable that we did not find any 9.6^- $D66^+$ cell population. However, we did find a few cases with 9.6^+ cells that did not form rosettes with E_{AET} and that could not be included in the present study because of lack of cells.

Could some $D66^-$ or low D66 surface density lymphoblastoid cells have hidden D66 epitopes? As indicated in Table V, we treated cells from four patients with neuraminidase. Three patients had 9.6^+, low density D66 cells. After neuraminidase

TABLE IV

Cocapping and Lysostripping of 9.6 and D66 on Thymocytes*

First antibody	Percent cells forming cap with 1st Ab	Percent cells stained but not capped with 1st Ab	Second antibody	Percent of cells capped by the 1st Ab restained by the 2nd Ab		Percent of cells capped by the 1st Ab lysed after adding 2nd Ab + C'
				1st cycle capping	2nd cycle capping	
9.6	52 ± 10	20 ± 4	9.6	10	4	6
			Anti-D.66	30	11	10
			W6.32‡	43	40	28
Anti-D66	39 ± 7	29 ± 5	Anti-D66	80	50	9
			9.6	80	61	7
			W6.32	80	80	26

* Thymocytes were incubated for 20 min with an excess amount of the 1st MAb. After washings, thymocytes were incubated for 45 min at 37°C with an excess amount of fluoresceinated goat anti-mouse Ig (one cycle). After further washings, they were incubated for 5 min at room temperature with pure mouse ascites induced by MOPC 21 NSI/I plasmocytoma cell line. They were next incubated for 30 min at room temperature with an excess amount of the 2nd MAb. After washings, they were incubated for 30 min at room temperature with an excess amount of TRITC-F(ab')₂ goat-anti-mouse Ig. For lysostripping experiments, the capping cycle was performed as described above. The cells were then processed as described in ref. 43.
‡ W6.32 is a monomorphic anti-HLA MAb.

treatment, D66 density increased as these cells acquired the property to form 37°C stable E-rosettes. One patient had 9.6⁻, D66⁻ cells. After neuraminidase treatment, D66 and 9.6 densities did not increase, nor did these cells acquire the ability to form E-rosettes.

Discussion

The major conclusion of our present study is that D66 is an epitope involved in the interactions between T lymphocytes and E. First, we observed that anti-D66 is able to block E-rosette formation both at 4° and 37°C in a dose-dependent manner until complete inhibition. However, when E were pretreated with AET or neuraminidase, we observed no blocking effect regardless of the amount of anti-D66 we added. Second, after we could induce an almost complete displacement of D66 on the cell surface, the T cells lost their ability to form E-rosettes. Thus, the D66 epitope or a closely related epitope carried by the same molecule is directly involved in the interactions between T cells and E.

This epitope is clearly different from the epitope defined by the MAb 9.6 obtained by Kamoun et al. (13). We observed no cross-blocking between the fixation of anti-D66 and 9.6. Yet, both epitopes are carried by the same molecule of 50,000 mol wt, as revealed by sequential immunoprecipitation assays on two different T cell lines. On thymocytes, also, the majority of both epitopes appeared to be carried by the same molecule, as this is consistent with the results of cocapping and lysostripping experiments. In addition, a large fraction of D66 was hidden (Fig. 7) or prevented from reacting with anti-D66 by sialic acid, whereas neuraminidase treatment of T cells produced no change in reactivity with 9.6. Although we shall further discuss
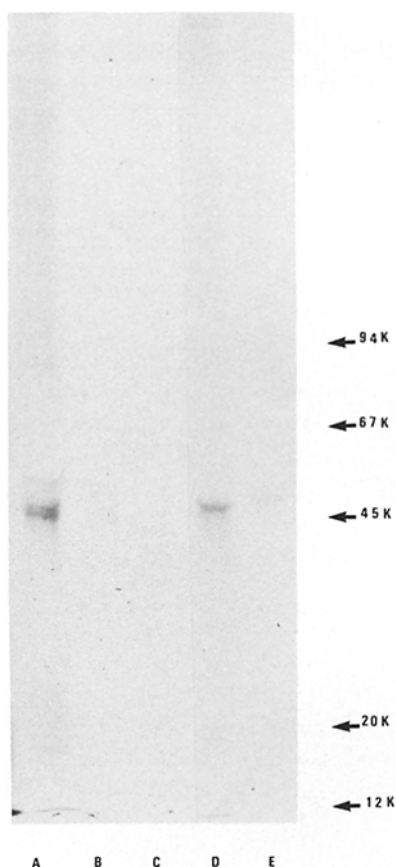
FIG. 6. Radioimmune precipitation assays with 9.6. and anti-D66 of the leukemic cell line HDMAR. Cells were radiolabeled with [^{35}S]methionine and immunoprecipitated as indicated in Materials and Methods. Analysis was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The positions of migration of the molecular weight markers are indicated by arrows. (A) immunoprecipitation with anti-D66; (B) anti-D66 after a first precipitation with anti-D66; (C) immunoprecipitation with 9.6. after precipitation with anti-D66; (D) immunoprecipitation with 9.6.; and (E) immunoprecipitation with anti-D66 after precipitation with 9.6. Identical results were obtained using another cell line (KE 37).

evidence for D66 being a hidden epitope, such concealed structures on lymphocytes have been clearly demonstrated (28, 29).

As on E$^+$-PBL, the D66 density is much lower than on thymocytes, even though the cells have been treated with neuraminidase; and as 9.6 surface density on E$^+$-PBL is similar to 9.6 density on thymocytes, it is likely that many 9.6$^+$, D66$^-$ molecular variants would be present on E$^+$-PBL. However, the entire population of peripheral T cells displays both 9.6 and D66, as this is apparent from fluorometer histograms.

We observed a striking correlation between the surface density of D66 of a given T cell population and its ability to form stable rosettes at 37°C. Thus, D66 density is high on the surface of the vast majority of thymocytes, T cells from tonsils and adenoids, T lymphoblasts after allogeneic stimulation or stimulation with PHA, and, significantly more, after stimulation with Con A. All these T cell populations are known to form stable E-rosettes at 37°C (30, 37). Moreover, the kinetic of increase in

TABLE V

Comparisons between the Ability of T Lymphoblastoid Cell Populations to Form Various Types of E-Rosettes and Their Surface Densities in 9.6 and D66 Epitopes

Lymphoblastoid cells*	E-rosette‡ formation			Reactivity with MAb§		
	E_{AET}	E_4	E 37°C	9.6	D66, fluoresceinated	C′ cytotoxicity
Cell lines						
HDMAR	90	71	51	+++	+++	+
KE37	95	92	60	++	++	+
JM	40	37	0	++	−	+
MOLT4	5	0	0	−	−	−
CEMT	0	0	0	−	−	−
HSB2	22	1	0	−	−	−
Peer	12	0	1	−	−	−
T cell malignancies						
T-LL$_1$	91	90	61	+++	++	+
T-LL$_2$	85	80	66	++	+++	+
T-LL$_3$	91	91	71	++	+++	+
T-LL$_4$	92	79	89	++	++	+
T-LL$_5$	97	78	84	++	++++	+
T-ALL$_1$	80	56	31	+++	+	+
T-LL$_6$	80	54	15	++	−	+
Séz$_1$	70	40	8	+++	−	+
T-ALL$_2$	75	NT‖	<1	+++	−	+
T-LL$_7$	74	22	<1	++	−	+
Séz$_2$	58	19	13	+++	−	+
T-LL$_8$	22	11	<1	+	−	±
T-ALL$_3$	8	4	<1	−	−	−
T-ALL$_4$	10	<1	<1	−	−	−
T-ALL$_5$	7	<1	<1	−	−	−
T-ALL$_6$	<1	<1	<1	−	−	−
After neuraminidase treatment						
T-LL$_6$	84	78	49	++	+	NT
Séz$_1$	82	56	36	+++	+	NT
Séz$_2$	70	40	19	+++	+	NT
T-ALL$_3$	7	4	<1	−	−	−

* 9.6⁻, E⁻ lymphoblastoid cell populations were classified as T cells because they reacted with several hetero-anti-T cell antisera (19) and monoclonal antibodies defining T cell specific epitopes. For instance, T-ALL 3-6 had cells that reacted with anti-T6 (24) and D47 (25) and 3A1 (26); they lacked cytoplasmic or surface Ig, HLA-DR, nor did they react with anti-B1 MAb (27).

‡ Percent of E-rosette-forming cells morphologically identified after sedimentation and Wright's staining.

§ 9.6, +++ is the density on both thymocytes and E⁺-PBL; D66, +++ is the density on thymocytes; +, density on E⁺-PBL.

‖ Not tested.

D66 densities on the surface of T cells after mitogenic stimulation was identical to the kinetic of increase in their ability to form 37°C stable E-rosettes. Similarly, treatment of E⁺-PBL with an appropriate amount of neuraminidase increased both the density of D66 and the number of 37°C stable E-rosettes (8). Limited enzymatic destruction decreased D66 surface density and the thymocytes' ability to form 37°C stable E-rosettes in a parallel manner. More strikingly, we investigated 7 T lymphoblastoid
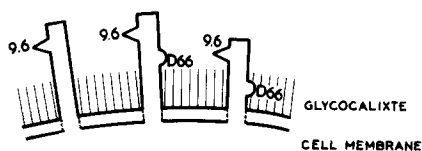
Fig. 7. A view of the T cell molecule responsible for interactions with sheep E.

cell lines in long-term culture and 16 cases of T lymphoid malignancies and compared their surface densities in 9.6 and D66 epitopes with their aptitude to form rosettes with E_{AET} and untreated E at 4°C and at 37°C. The results we obtained were clear-cut: we found no lymphoblastoid T cell population displaying D66 that did not also display 9.6. The T lymphoblastoid cell populations displaying 9.6 and low densities of D66 formed rosettes with E_{AET} or, to a lesser extent, with untreated E at 4°C, but no significant number of E-rosettes at 37°C. The T lymphoblastoid cell populations that carried high densities of both D66 and 9.6 formed stable rosettes at 37°C.

Therefore, it is possible to see the T cell surface structure responsible for interacton with E as follows: the 9.6 epitope is necessary for stable interaction between T cells and E. When E are pretreated with AET or neuraminidase, such interaction is sufficient to permit rosette formation and stability, regardless of temperature, either because the number of structures interacting with 9.6 on the E surface has been increased or because they have been modified so that the affinity of interaction would have increased. When E are not treated, the D66 epitope is also required for rosette formation. The interaction between D66 and anti-D66 structure on E would have maximum affinity at 4°C, so that when the density of D66 is low, rosette stability is ensured only at 4°C. In contrast, when D66 density is high, rosette stability is also ensured at 37°C. On the thymocytes and T blast surfaces, most of the molecules carry both D66 and 9.6, whereas on E^{+}-PBL, most of the molecules would carry the 9.6 epitope only.

In no way do we pretend that this view could account for all the events leading to rosette formation: most probably other structures, possibly other molecules, should be involved in interactions between E and T cells, as, for instance, the active E-rosettes test appears to mark a population differing from the E-rosettes obtained at 37°C (2, 23); yet, both assays are blocked by 9.6 and anti-D66. It is also noteworthy that we could observe, among T cell malignancies, few cases with cells displaying 9.6, but that were unable to form rosettes with E_{AET} (these results could not be included in the present study because of lack of cells) (14, 15).

While performing our investigations, we were surprised by two phenomena: (a) although we could obtain capping of D66 as revealed by fluorescence gathering, relabeling of the cells with anti-D66 showed that, after such a capping, important amounts of diffuse D66 were then present on the cell surface, and (b) blocking of E-rosette formation by anti-D66 was totally raised after fixation of a second antibody on anti-D66. Actually, an Fab fragment of anti-mouse Ig (Fab-MIg) could both raise the E-rosette inhibition produced by anti-D66 and induce further appearance of D66 on the thymocyte surface.

We found the effects of Fab-MIg to be reminiscent of TL molecule modulation, which can be induced by Fab fragments, and by which a cell can escape C'-dependent lysis, although TL-anti-TL complexes are still present on the cell surface (32–35).

This appears to be a complex phenomenon, involving several mechanisms, but the basic event would be a microdisplacement of TL molecules induced by fixation of anti-TL Ig, even though it would be limited to an Fab fragment (33). Dealing with the D66-carrying molecule, this microdisplacement would cause further rearrangement of all these molecules, including those carrying hidden D66, perhaps because of their involvement in complex supermolecular organization on the cell surface (L. Boumsell and A. Bernard, manuscript in preparation).

It is known that quite a number of T cell malignancies have malignant cell populations that do not form rosettes with E (21, 36, 37). Pretreating E with AET was shown to be the most efficient technique for detecting the ability of a malignant T cell population to form E-rosettes (38). This inability to form E-rosettes was demonstrated for Sézary cells as well as leukemic and lymphomatous cells, with characteristics of T cells quite advanced along the T cell maturation process, both in terms of surface antigens and functions (39–42). We show here that the treatment of such malignant T cell populations with neuraminidase uncovers in many cases the otherwise masked D66 epitope. This treatment also confers to these cells the ability to form rosettes with untreated E at 37°C. In other cases, particularly when the 9.6 epitope is not readily detectable, no D66 is uncovered after neuraminidase treatment, nor are these malignant cells conferred the ability to form E-rosettes. Thus, abnormalities of the glycocalixte of malignant T cells can induce abnormalities of their surface antigenic phenotype.

The events we have called D66 "modulation" on the thymocyte surface should be related to the functional role of the molecule. Thus, modulation of cell surface structures should be a widely shared property of such structures, whose triggering could be the privilege of preculiar epitopes. Although having large physiological significance, such events could lead to escape of immune destruction when occurring on the surface of malignant cells (44–46).

Summary

Anti-D66 is a monoclonal antibody able to inhibit E-rosette formation of T cells both at 4°C and at 37°C but that does not inhibit T cell rosette formation with neuraminidase or 2-amino-ethylisothiouronium bromide (AET)-pretreated E. As demonstrated by capping experiments, it defines an epitope, D66, that is directly involved in E-rosette formation. D66 is distinct from the epitope defined by 9.6 because 9.6, a previously defined "pan-T" monoclonal antibody, inhibits E_{AET} rosette formation and because no cross-blocking occured between both antibodies fixation. However, 9.6 and D66 are carried by the same molecule, as demonstrated by sequential immunoprecipitation assays performed on two different T cell lines. On the thymocyte surface, also, 9.6 and D66 are most probably carried by the same molecule, as indicated by cocapping and colysostripping experiments. D66 is present at higher densities on thymocytes and activated T cells than on peripheral blood T cells. Investigation of numerous T cell populations, both normal and malignant, showed a straightforward correlation between elevated D66 density and ability to form 37°C stable E-rosettes. Neuraminidase treatment of thymocytes and peripheral blood lymphocytes forming E-rosettes unmasked a large fraction of D66 not readily accessible on their surface. These hidden D66 epitopes appear to be responsible for a surprising observation: the ability of anti-D66 to inhibit E-rosette formation could be

totally reversed by fixation on anti-D66 of an antibody to mouse immunoglobulin or an Fab fragment anti-mouse immunoglobulin. This would induce microdisplacement with emergence of hidden D66, as documented by fluorometric studies. Finally, malignant T cells with a differentiative status of mature T cells, but forming no (or low numbers of) E-rosettes, could be induced both to display D66 and to form E-rosettes by neuraminidase treatment.

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