

## IDIOTYPIC DETERMINANTS ON T-CELL SUBPOPULATIONS\*

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Specific immunocompetent lymphocytes normally function within the immune society as social members under control by other lymphoid cells. Such controlling signals may be of specific or nonspecific nature and may lead to stimulation or inhibition of the cell to be regulated. Of particular importance in this context are the T lymphocytes, in which several distinct subgroups of cells as defined by morphology, function, or specificity have been identified (1).

The specificity of the immunocompetent T lymphocyte is known to be an inherent property of the cell, but distinct subgroups of T cells would seem to recognize foreign structures via fundamentally different pathways. It is thus clear in the mouse that whereas one subgroup, the so-called helper T cells normally "see" antigen in the context of Ia antigens, killer T cells would normally recognize foreign structures in association with H-2K or D molecules (1-6). Suppressor T cells as a third category behave as if they are able to react with soluble antigen in a manner more analogous to B lymphocytes and conventional antibodies (7). In the murine system it has also been possible to associate T-cell function with distinct surface alloantigens in a manner so far not possible in other species. Thus, helper T cells are normally of the antigenic phenotype  $\text{Lyt-1}^+2^-3^-$ , whereas killer T cells behave as if they are predominantly  $\text{Lyt-1}^-2^+3^+$  (1, 8). It is still debatable, however, whether this surface structure distinction is absolute or whether some anti-Ia reactive cells may be of "killer T type" according to Lyt phenotype(s) and similarly whether some anti-H-2K/D reactivity may reside in  $\text{Lyt-1}^+2^-3^-$  cells.

One successful way of studying the antigen-binding receptors of T lymphocytes has been to employ anti-idiotypic reagents (9, 10). By using such an approach it has thus been possible to demonstrate the existence of idiotypic or anti-idiotypic receptors on the surface of helper and mixed leukocyte culture (MLC)-reactive<sup>1</sup> cells as well as on suppressor T cells. Idiotypic receptors on killer T cells have so far only been described in an indirect way, namely, by the induction of alloantigen-specific cytolytic T cells with anti-idiotypic antibodies as specific inducing agents (11).

In the present article we describe results proving the existence of idiotypic receptors on effector killer T cells in the rat with specificity for Ag-B antigens, and in the mouse with specificity for H-2 antigens. Furthermore, we have analyzed the distribution

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<sup>1</sup> Abbreviations used in this paper: CML, cell-mediated lympholysis; D-PBS, Dulbecco modified phosphate-buffered saline ( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  free); EHAA, Eagle's high amino acid; FCS, fetal calf serum; [<sup>3</sup>H]TdR, tritiated thymidine; LPS, bacterial lipopolysaccharide; MLC, mixed leukocyte culture.

pattern of idiotypes on Lyt-1<sup>+</sup>2<sup>-</sup>3<sup>-</sup> or Lyt-1<sup>-</sup>2<sup>+</sup>3<sup>+</sup> T lymphoblasts obtained from the same MLC culture across an entire H-2 barrier with Lyt-purified blasts either as autoimmunogen to produce specific unresponsiveness via autoanti-idiotypic immunity (12) or as specific absorbants of anti-idiotypic antibodies that can eliminate MLC or cell-mediated lympholysis (CML) reactivity. Our results strongly suggest, at least in the present antigenic systems, that the two subgroups of T lymphocytes may indeed express entirely distinct spectra of idiotypes. Finally, we have now extended previous observations of the triggering of allospecific killer T cells by autoanti-idiotypic antibodies to include study of the triggering of Lyt-purified T-cell subsets. Data will be presented which indicate that anti-idiotypic antibodies may serve as a more potent triggering signal than the actual allogeneic cells themselves, and may actually obviate the need for Lyt-1<sup>+</sup>2<sup>-</sup>3<sup>-</sup> T cells in the primary induction of cytotoxic T cells in the mouse.

### Materials and Methods

*Animals.* (a) Mice: Mice of the inbred strains CBA/J, C57BL/6J, DBA/2J, and BALB/c were purchased from Bomholtgård Ltd., Ry, Denmark, and (or) bred and maintained in our own colony. Mice were between 5 and 10 wk old when used for experiments. (b) Rats: Rats of the inbred strains Lewis (Ag-B<sup>1</sup>), DA (Ag-B<sup>4</sup>), BN (Ag-B<sup>3</sup>), as well as F<sub>1</sub> animals between these strains, were bred and maintained in our own colony. Young adult rats were used within the experiments.

*Cell Preparations.* Spleens were aseptically removed, and single-cell suspensions were prepared by using a stainless steel mesh and Dulbecco modified phosphate-buffered saline (D-PBS) as a medium. Large particles were removed by sedimentation and single cells were washed once in D-PBS. Erythrocytes were lysed by hypotonic shock with 0.9 ml of distilled water followed by 0.1 ml of D-PBS (concentrated 10 times) for five spleens. Lymphocytes were washed again and resuspended in culture medium. Dead cells were determined by trypan blue exclusion.

*Culture Medium.* The culture medium for lymphocyte cultures was Eagle's high amino acid medium (EHAA) (13), complemented with 0.5% normal fresh mouse serum,  $5 \times 10^{-5}$  2-mercaptoethanol, 100 IU/ml of penicillin, and 50  $\mu$ g/ml of streptomycin.

*MLC.* MLCs for analytical purpose were performed in flat-bottom microtiter plates (System Cook, 29 ART, Greiner, Nürtingen, Germany) with  $0.25 \times 10^6$  responder lymphocytes and  $0.5 \times 10^6$  2,500 rad irradiated stimulator cells. Triplicate or quadruplicate cultures were pulsed for 6 h with 1  $\mu$ Ci of [<sup>3</sup>H]trinitiated thymidine ([<sup>3</sup>H]TdR) (Radiochemical Centre, Amersham, England; 40–60 Ci/mmol sp act). Cultures were harvested on glass fiber paper with a Skatron multicell harvester (Skatron, SA, Lierbyen, Norway) and counted in 2 ml of scintillation fluid in plastic minivials with a Mark III liquid scintillation counter (model 6880, Searle Diagnostics Inc., Des Plaines, Ill.).

For the preparation of large quantities of specific T lymphoblasts, MLCs were performed in tissue culture flasks (model 3013; Falcon Plastics, Division of BioQuest, Oxnard, Calif.) with the same medium as described above. 15 ml of a suspension containing  $1.25 \times 10^6$  responder cells and  $2.5 \times 10^6$ /ml of 2,500 rad irradiated stimulator cells were added to each flask. Cultures were harvested on day 4 after initiation of the MLC (corresponding to peak response).

*CML.* CMLs were performed in V-bottom microtiter plates (Cook, M25 ART, Greiner, Nürting, Germany) in 200  $\mu$ l of EHAA medium complemented with 5% heat-inactivated (60 min, 56°C) fetal calf serum (FCS). Assays were carried out in triplicate or quadruplicate cultures with an effector to target cell ratio as indicated in the tables. Plates were incubated for 6 h at 37°C and 5% CO<sub>2</sub> in air. Percent cytotoxicity (% CML) is expressed as

$$100 \times \frac{\text{Experimental} - \text{spontaneous } ^{51}\text{Cr release}}{\text{Maximum} - \text{spontaneous } ^{51}\text{Cr release}}$$

The following target cells were used: bacterial lipopolysaccharide (LPS) blasts were induced from spleen cells from CBA, C57BL/6, BALB/c, or DBA/2 mice in 3013 tissue culture flasks.

Each flask contained 15 ml of a cell suspension consisting of  $3 \times 10^6$  lymphocytes/ml and 15  $\mu\text{g}$  LPS/ml (LPS was a gift from Dr. Jan Andersson (Institute for Immunology, Uppsala University, Uppsala, Sweden)). Blasts were harvested on day 3 of culture and purified on Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) as described below.  $5 \times 10^7$  blasts were labeled with 0.5 mCi of  $^{51}\text{Cr}$  (New England Nuclear Chemicals, Boston, Mass.) for 1.5 h at  $37^\circ\text{C}$  and washed five times thereafter. By such an approach between 0.5 and 0.8 cpm/cell was obtained. 100- $\mu\text{l}$  samples of the assay were counted in an Intertechnique gamma counter (model CG-4000; Intertechnique, SA, 78370 Plaisir, France), equipped with a 3 in. well-type crystal.

*Purification of MLC T Lymphoblasts and LPS Blasts.* Cultures were pooled in 50-ml Falcon tubes (Falcon No. 2070) and centrifuged for 10 min at 400 g. Cells were pooled and washed again in D-PBS and resuspended in D-PBS. Blasts were purified on Ficoll-Paque as described earlier (11). Blasts were washed three times in D-PBS.

*Preparation of T Lymphocytes.* Mouse and rat T lymphocytes were prepared from spleen cells by Ig-anti-Ig columns as described in detail earlier (14).

*Preparation of Anti-Idiotypic Antiserum of Anti-C57BL/6 Anti-CBA Specificity.* C57BL/6 mice were inoculated with purified C57BL/6 anti-CBA MLC T lymphoblasts. Each mouse received  $1 \times 10^7$  specific T lymphoblasts subcutaneously each time. Complete Freund's adjuvant was used for the first injection and incomplete adjuvant for the boosters. Animals received four injections in 3-wk intervals and were bled for the first time from the eye 2 wk after the fourth injection. Animals then received two more injections and were bled out 2 wk after the last injection. Sera were heat inactivated (30 min,  $56^\circ\text{C}$ ) and absorbed extensively with (C57BL/6  $\times$  CBA) $F_1$  spleen cells (1 ml of 1:2 diluted antiserum was absorbed three times with  $5 \times 10^7$   $F_1$  spleen cells). This absorption step was performed as we frequently found autoantibodies reactive with an unknown structure of all rat strain lymphocytes, as assessed by protein A technique (14). After absorption, however, the present antisera were not tested for remaining autoantibodies. Sera were then tested at a dilution of 1:10 for their specific inhibition of MLC by procedures described in detail elsewhere (14). Of 102 individual antisera, 5 were found to be capable of specifically inhibiting C57BL/6 anti-CBA MLC in a specific way, even at a dilution of 1:40. The response against DBA/2 stimulator cells was hardly touched. These five antisera also showed strong binding to C57BL/6 anti-CBA MLC T lymphoblasts as assessed by the protein A technique (14), but not to C57BL/6 anti-DBA/2 blasts. One out of these five antisera was also able to specifically inhibit CML at the same dilution by the aid of complement. The other 97 sera showed either no significant reduction of MLC responses or a nonspecific cytotoxic effect eliminating both responses against CBA as well as DBA/2.

Anti-(Lewis anti-DA) and anti-(Lewis anti-BN) were two anti-idiotypic antisera described in detail elsewhere. The first was antiserum 1003; its anti-idiotypic nature has been fully characterized by Binz and Wigzell (14). The latter antiserum is pool A which has been used and characterized (15).

*Treatment of T Lymphocytes with Anti-Lyt Antisera.* Anti-Lyt-1.2, 2.2, and 3.2 were produced at the Sloan-Kettering Cancer Center, New York, as described (16) or prepared in Uppsala in the following way: anti-Lyt-2.2 sera were produced in CE mice with weekly injections of  $10^7$  B10.BR thymocytes. Sera from individual mice were tested for specific antibodies after absorptions on CBA/H thymocytes to remove general autoantithymocyte antibodies. Anti-Lyt-1.2 antibodies were produced in a similar manner with C3H mice immunized with CE thymocytes. Specificity of anti-Lyt-antisera was verified by cytotoxic assays as indicated below on Lyt-congenic thymocytes. Anti-Lyt-1.2 antiserum was used at a final concentration of 1:30, anti-Lyt-2.2 at 1:40, and anti-Lyt-3.2 at 1:50. Anti-Lyt-2.2 and 3.2 were always used together.  $5 \times 10^7$  T cells were incubated with 1 ml of diluted antiserum for 30 min at room temperature, spun down, and the pellet resuspended in 1 ml of 1:8 diluted rabbit complement for 30 min at  $37^\circ\text{C}$  with occasional shaking. Cells were washed three times thereafter in D-PBS.

*Treatment of Lymphocytes with Anti-Idiotypic Antisera.*  $5 \times 10^7$  rat T lymphocytes were incubated with 0.5 ml anti-idiotypic antiserum. Serum 1003 was used at a final dilution of 1:20 and pool A at a final dilution of 1:10. After incubation for 1 h at  $4^\circ\text{C}$ , cells were washed once and rabbit complement was added at a final dilution of 1:7. Thereafter, cells were incubated for 30 min at  $37^\circ\text{C}$  and washed twice.

*Rat Fibroblast Monolayers.* Rat embryos from Lewis, DA, and BN strains were removed from

their mothers as soon as pregnancy was detectable. Heads, legs, and intestines were removed and the rest cut into small pieces and trypsinized for 15 min at room temperature in 0.25% trypsin containing 0.03% EDTA. Trypsinized cells were harvested and washed twice in RPMI 1640 containing 10% FCS. 1 ml of a cell suspension containing  $4 \times 10^7$  viable cells was added to 20 ml of RPMI medium complemented as described above in a 3024 Falcon tissue culture flask. On the next day the attached cells were washed once and fed twice a week. Two passages were carried out in weekly intervals, the cells from one flask being divided into three new flasks. Rat fibroblasts to be used as targets for cytolytic T cells were cultured in flat-bottom microtiter flasks (Falcon 3040). 1,000 cells from the second passage were added per well and cultured until a complete monolayer was detectable. Such monolayers were used as targets (see CML).

*Cytolysis on Rat Fibroblast Monolayers (CML).* Cytolytic T lymphocytes were elicited on rat fibroblast monolayers in Falcon 3024 tissue culture flasks. Rat T lymphocytes were purified on Ig-anti-Ig columns from spleens and lymph nodes. Before culture, T cells were incubated with different antisera as shown in Table I.  $1 \times 10^8$  treated rat T lymphocytes were added to each flask containing rat fibroblast monolayer in 20 ml RPMI 1640 containing 10% FCS. T cells (Lewis) were incubated with either (Lewis  $\times$  DA) $F_1$  normal serum or serum 1003 and complement before culture, and then cultured on either DA or BN rat fibroblast monolayers (see Table I). In the same way DA T cells were incubated with (Lewis  $\times$  DA) $F_1$  normal serum or serum 1003 and complement and cultured on Lewis fibroblasts. On day 7 of culture, surviving T lymphocytes were removed, washed once in D-PBS, and tested for their lytic activity on different  $^{51}\text{Cr}$ -labeled fibroblast monolayers in flat-bottom microtiter plates. Effector cells were either untreated or treated with normal  $F_1$  antisera or anti-idiotypic antisera 1003 or pool A and complement.

Rat fibroblast monolayers in Falcon flat-bottom microtiter wells were labeled with 0.05  $\mu\text{Ci}$  of  $^{51}\text{Cr}$  in 100  $\mu\text{l}$  RPMI 1640/well for 4 h at 37°C. Fibroblasts were washed six times and  $1 \times 10^6$  treated or untreated effector cells in 200  $\mu\text{l}$  of medium containing 10% heat-inactivated FCS (60 min at 56°C) were added per well and incubated overnight. Plates were centrifuged for 5 min at 200 g, and 100  $\mu\text{l}$  of the supernate was counted. Maximal release was determined by incubation with 200  $\mu\text{l}$  of 0.2% sodium dodecyl sulfate.

## Results

*1. Specific Inhibition of Rat Cytolytic T Lymphocytes by Anti-Idiotypic Antibodies.* We have previously shown (14) that anti-idiotypic antibodies raised against T lymphocyte receptors with a given specificity can inhibit the MLC response of corresponding specificity by the aid of complement. Thus, an antiserum of anti-(Lewis anti-DA) specificity can specifically inhibit Lewis anti-DA MLC and an antiserum of anti-(Lewis anti-BN) specificity can inhibit the Lewis anti-BN response. Third-party reactivity is either not affected or only slightly. We asked the question whether such antisera might also inhibit cytolytic T lymphocytes at the effector phase and(or) the generation of cytolytic T lymphocytes. Two anti-idiotypic antisera have been used: one of anti-(Lewis anti-DA) specificity (serum 1003 used in the experiments in reference 14) and one with anti-(Lewis-anti-BN) specificity (serum pool A used in the experiments in reference 15). Cytolytic rat T lymphocytes were generated on rat fibroblast monolayers as described in Materials and Methods. The experiments are shown in Table I.

Lewis T lymphocytes were incubated with (Lewis  $\times$  DA) $F_1$  normal rat serum and complement or with anti-idiotypic antiserum of anti-(Lewis anti-DA) or anti-(Lewis anti-BN) specificity and complement. This treatment was performed at the initiation of in vitro incubation or at the effector cell stage (see Table I). Lymphocytes were incubated on allogeneic rat fibroblasts (either DA or BN) for 7 d. Surviving cells were harvested and used directly as effector cells or incubated with either normal  $F_1$  serum or anti-idiotypic antisera. Such treated cells were then added to  $^{51}\text{Cr}$ -labeled fibroblast

TABLE I  
*Anti-Idiotypic Antisera Can Specifically Inhibit the Generation of Specific Killer T Lymphocytes As Well As the Activity of Killer T Cells*

Lymphocytes from	Treated with	Cultured on	Effector cells	Percent lysis on Fibroblasts		
				DA	BN	Lewis
				%		
Lewis	(L × DA)F <sub>1</sub> -NRS + C'*	DA fibroblasts	—	67.3	—	—
Lewis	Anti-(L anti-DA) + C'	DA fibroblasts	—	4.0	—	—
Lewis	(L × DA)F <sub>1</sub> -NRS + C'	DA fibroblasts	(L × DA)F <sub>1</sub> -NRS + C'	45.0	—	—
Lewis	(L × DA)F <sub>1</sub> -NRS + C'	DA fibroblasts	Anti-(L anti-DA) + C'	0.6	—	—
Lewis	(L × DA)F <sub>1</sub> -NRS + C'	DA fibroblasts	Anti-(L anti-BN) + C'	60.0	—	—
Lewis	(L × DA)F <sub>1</sub> -NRS + C'	BN fibroblasts	—	—	41.2	—
Lewis	Anti-(L anti-DA) + C'	BN fibroblasts	—	—	32.9	—
Lewis	(L × DA)F <sub>1</sub> -NRS + C'	BN fibroblasts	(L × DA)F <sub>1</sub> -NRS + C'	—	31.8	—
Lewis	(L × DA)F <sub>1</sub> -NRS + C'	BN fibroblasts	Anti-(L anti-DA) + C'	—	30.9	—
Lewis	Anti-(L anti-DA) + C'	BN fibroblasts	Anti-(L anti-DA) + C'	—	33.0	—
Lewis	(L × DA)F <sub>1</sub> -NRS + C'	BN fibroblasts	Anti-(L anti-BN) + C'	—	0.1	—
DA	(L × DA)F <sub>1</sub> -NRS + C'	L fibroblasts	—	—	—	72.1
DA	Anti-(L anti-DA) + C'	L fibroblasts	—	—	—	63.7
DA	(L × DA)F <sub>1</sub> -NRS + C'	L fibroblasts	(L × DA)F <sub>1</sub> -NRS + C'	—	—	57.0
DA	(L × DA)F <sub>1</sub> -NRS + C'	L fibroblasts	Anti-(L anti-DA) + C'	—	—	55.5

Lewis and DA T lymphocytes were treated with different antisera as indicated and cultured for 7 d on the rat fibroblast monolayers as indicated. Cytolytic T lymphocytes were harvested and left untreated or treated with different sera as indicated. Such T-effector cells were then incubated overnight with <sup>51</sup>Cr-labeled fibroblasts in flat-bottom microtiter plates. 1 × 10<sup>6</sup> effector cells were used per microtiter plate well (for details see Materials and Methods).

*Abbreviations used:* NRS, normal rat serum; anti-(Lewis anti-DA), anti-idiotypic antiserum 1003 (14); anti-(Lewis anti-BN), anti-idiotypic antiserum pool A (15); L, Lewis.

monolayers in flat-bottom microtiter plates. The results were quite clear cut. Anti-idiotypic antisera could specifically inhibit both cytolytic effector T lymphocytes as well as the actual generation of specific killer T cells, indicating that both cell types must display idiotypic surface determinants with supposedly similar idiotypes. The same antisera had no effect on DA rat T lymphocytes activated against Lewis fibroblasts, nor were they active against Lewis T cells of irrelevant specificity.

2. *Autoimmunization with Alloreactive T-Cell Subpopulations.* The experiments on rat killer T cells were then extended to murine cytolytic T cells. In a first series of experiments we immunized C57BL/6 mice with Lyt-1<sup>+</sup>2<sup>-</sup>3<sup>-</sup> or Lyt-1<sup>-</sup>2<sup>+</sup>3<sup>+</sup> T cells purified out of specifically activated C57BL/6 anti-CBA MLC T lymphoblasts. MLC T lymphoblasts were harvested on day 4 after initiation of the MLC and purified on Ficoll-Paque as described in Materials and Methods. C57BL/6 anti-CBA "helper" cells were obtained by incubation of the MLC T lymphoblasts with anti-Lyt-2,3 and complement and "killer" cells with the same specificity by incubation with anti-Lyt-1 and complement as described in Materials and Methods. Each animal received 2 × 10<sup>7</sup> purified T cells subcutaneously (either helper or effector cells) emulsified in complete Freund's adjuvant. Injections were repeated three times in 3- or 4-wk intervals with the same number of cells emulsified in incomplete Freund's adjuvant. Animals were sacrificed 2 mo after the last injection and their spleen cells individually used for the preparation of MLCs. The proliferative response was measured on day 4

of culture against CBA and BALB/c stimulator cells. The cytolytic response (CML) was measured on day 6 of culture against CBA and BALB/c targets. The results are summarized in Table II.

Four normal C57BL/6 mice that had been immunized with Freund's adjuvant only were used as positive controls for the MLC and CML response against CBA and BALB/c targets, and the mean of these individual values was considered as 100% responses (for details, see legend to Table II). Eight C57BL/6 mice were immunized four times with either Lyt-1<sup>+</sup> cells or with Lyt-2<sup>+</sup>3<sup>+</sup> cells. Mice autoimmunized with Lyt-1<sup>+</sup>2<sup>-</sup>3<sup>-</sup> cells showed a remarkable reduction of the MLC response against CBA alloantigens (about 28% of the response of the control value), whereas the response against BALB/c stimulator cells was only slightly touched (about 77% of the control value). At the same time the CML response against CBA targets was strongly suppressed (about 22% of the control response), but lytic activity against BALB/c targets was not reduced (remaining activity about 96% of the control).

In contrast, the eight mice autoimmunized with Lyt-2<sup>+</sup>3<sup>+</sup> effector cells were slightly reduced in the proliferative response against CBA stimulator cells (about 77% of the control response) and a normal response against BALB/c stimulators (about 93% of the control). The CML response against CBA alloantigens, however, was strongly suppressed (about 22% of the control value), whereas cytolytic reactivity against BALB/c was not touched.

In summary, autoimmunization of C57BL/6 mice with C57BL/6 anti-CBA Lyt-1<sup>+</sup>2<sup>-</sup>3<sup>-</sup> T lymphoblasts results both in a specific suppression of the MLC response as measured by [<sup>3</sup>H]TdR incorporation and in a failure in the generation of cytolytic T lymphocytes with specificity for CBA SD antigens. On the other hand, autoimmunization of C57BL/6 mice with C57BL/6 anti-CBA Lyt-1<sup>-</sup>2<sup>+</sup>3<sup>+</sup> cells fails to influence the proliferative response but results in a significant reduction in the generation of cytotoxic T lymphocytes of C57BL/6 anti-CBA specificity.

3. *Helper and Effector T Lymphocytes Bear Different Groups of Idiotypic Determinants.* The results described in section 2 above suggested that distinct groups of idiotypic determinants may exist on Lyt-1<sup>+</sup>2<sup>-</sup>3<sup>-</sup> helper T cells compared with those present on the Lyt-1<sup>-</sup>2<sup>+</sup>3<sup>+</sup> killer T cells. To elucidate this problem we have performed the following experiments. A strong anti-idiotypic antiserum of C57BL/6 anti-(C57BL/6 anti-CBA) specificity was selected out of 102 individual mouse antisera (see Materials and Methods). This serum could be shown to inhibit, in a specific way, both MLC and CML activity by the aid of complement. The antiserum was then absorbed with either C57BL/6 anti-CBA "helper" cells (Lyt-1<sup>+</sup>2<sup>-</sup>3<sup>-</sup> cells) or C57BL/6 anti-CBA "effector" cells (Lyt-1<sup>-</sup>2<sup>+</sup>3<sup>+</sup> cells). 1 ml of 1:30 diluted antiserum was thus absorbed three times for 30 min at 4°C with  $1 \times 10^8$  purified T cells of indicated subpopulation. The absorbed antiserum was then sterile filtered and used to inhibit either MLC or CML by procedures described in detail elsewhere (14). The results are shown in Table III.

Normal C57BL/6 serum from mice that have been immunized with Freund's adjuvant only served as control serum. MLC and CML responses of C57BL/6 spleen cells treated with control serum and complement (measured on day 4 for MLC and on day 6 for CML) against CBA and DBA/2 targets were considered to be 100%. The response of the very same cells incubated with the anti-idiotypic antiserum and complement was specifically reduced. The MLC response against CBA stimulators

TABLE II  
Autoimmunization of C57BL/6 Mice with Alloantigen-Activated T-Cell Subpopulations

Responder cells derived from individual animals	MLC responders				CML responses					
	CBA	Mean $\pm$ SE	BALB/c	Mean $\pm$ SE	Responder cells alone	Mean $\pm$ SE	CBA	Mean $\pm$ SE	BALB/c	Mean $\pm$ SE
<b>Normal C57BL/6</b>		%		%		%		%		%
1	240,231 $\pm$ 16,879		323,559 $\pm$ 40,237		1,477 $\pm$ 422		60.5 $\pm$ 3.8		49.1 $\pm$ 5.4	
2	212,862 $\pm$ 29,333		301,794 $\pm$ 33,454		1,949 $\pm$ 385		75.3 $\pm$ 1.5		91.4 $\pm$ 0.6	
3	285,442 $\pm$ 7,292	(100)	326,061 $\pm$ 27,480	325,903 (100)	1,929 $\pm$ 496	1,790 (100)	74.1 $\pm$ 7.5	72.4 (100)	85.1 $\pm$ 2.2	76.1 (100)
4	276,030 $\pm$ 15,833		352,197 $\pm$ 2,423	$\pm$ 10,320	1,805 $\pm$ 246	$\pm$ 109	76.5 $\pm$ 1.2	$\pm$ 4.0	78.9 $\pm$ 0.7	$\pm$ 9.4
<b>C57BL/6 autoimmunized with Lyt-1<sup>+</sup> cells</b>		%		%		%		%		%
1	20,310 $\pm$ 6,939		284,621 $\pm$ 26,951		6,202 $\pm$ 742		14.4 $\pm$ 1.6		60.3 $\pm$ 3.7	
2	26,433 $\pm$ 275		274,099 $\pm$ 27,407		10,581 $\pm$ 82		15.1 $\pm$ 3.1		79.8 $\pm$ 2.0	
3	45,215 $\pm$ 4,032		248,526 $\pm$ 2,569		3,979 $\pm$ 522		10.4 $\pm$ 2.1		58.8 $\pm$ 1.0	
4	124,101 $\pm$ 11,005		244,496 $\pm$ 8,992	252,806 (71.57)	3,895 $\pm$ 80	5,949 (332.35)	19.3 $\pm$ 4.0	15.73 (21.73)	76.5 $\pm$ 2.2	73.21 (96.20)
5	127,406 $\pm$ 17,694	(28.41)	279,254 $\pm$ 10,277	$\pm$ 9,362	7,200 $\pm$ 253	$\pm$ 826	18.5 $\pm$ 4.0	$\pm$ 1.25	75.7 $\pm$ 2.4	$\pm$ 3.11
6	63,613 $\pm$ 2,877		257,958 $\pm$ 4,793		4,479 $\pm$ 256		14.9 $\pm$ 1.0		74.8 $\pm$ 5.1	
7	57,932 $\pm$ 7,563		220,533 $\pm$ 25,252		4,102 $\pm$ 71		12.5 $\pm$ 1.3		77.1 $\pm$ 6.1	
8	111,565 $\pm$ 3,284		212,959 $\pm$ 13,248		7,152 $\pm$ 170		20.7 $\pm$ 2.8		82.7 $\pm$ 1.2	
<b>C57BL/6 autoimmunized with Lyt-2.3<sup>+</sup> cells</b>		%		%		%		%		%
1	138,228 $\pm$ 23,870		316,750 $\pm$ 22,043		2,585 $\pm$ 700		14.1 $\pm$ 3.9		73.1 $\pm$ 6.0	
2	172,719 $\pm$ 22,395		271,862 $\pm$ 17,178		5,902 $\pm$ 1,478		20.0 $\pm$ 2.1		79.0 $\pm$ 9.0	
3	184,581 $\pm$ 26,497		364,903 $\pm$ 7,634		2,719 $\pm$ 66		19.5 $\pm$ 3.7		83.8 $\pm$ 2.4	
4	198,398 $\pm$ 16,341		259,912 $\pm$ 20,438	303,030 (92.98)	6,852 $\pm$ 2,176	4,251 (237.49)	28.9 $\pm$ 3.7	22.54 (31.13)	73.0 $\pm$ 2.3	77.61 (101.98)
5	198,329 $\pm$ 24,507	(77.42)	286,835 $\pm$ 29,130	$\pm$ 13,040	4,273 $\pm$ 283	$\pm$ 538	37.2 $\pm$ 5.2	$\pm$ 2.65	86.9 $\pm$ 1.4	$\pm$ 2.37
6	280,274 $\pm$ 35,290		347,588 $\pm$ 13,243		3,296 $\pm$ 435		17.3 $\pm$ 1.4		73.0 $\pm$ 1.5	
7	170,688 $\pm$ 13,994		288,489 $\pm$ 12,562		4,780 $\pm$ 422		24.9 $\pm$ 1.0		83.8 $\pm$ 3.4	
8	227,764 $\pm$ 7,578		287,902 $\pm$ 27,032		3,604 $\pm$ 468		18.4 $\pm$ 1.2		68.3 $\pm$ 1.3	

C57BL/6 mice were autoimmunized four times with either  $2 \times 10^7$  C57BL/6 anti-CBA helper T cells or C57BL/6 anti-CBA effector T cells. Lymphocytes were administered subcutaneously, emulsified in Freund's adjuvant. Complete Freund's adjuvant was used for the first injection and incomplete adjuvant for the boosters. Helper and effector cells were purified as described in Materials and Methods. Animals were killed 2 mo after the last injection, and their spleens used individually for the induction of MLC and CML responses against alloantigens as mentioned in the table. MLC responses were measured on day 4 and CML responses on day 6 of culture. For details, see Materials and Methods. Normal control animals were injected with Freund's adjuvant only. MLC and CML responses of these animals were considered to be 100%.

TABLE III  
*Alloantigen-Reactive Helper and Effector T Lymphocytes Bear Different Idiotypes*

Serum	MLC response			CML response	
	CBA	DBA/2	Responder cells alone	CBA	DBA/2
	%	%	%	%	%
Normal C57BL/6	293,052 ± 25,908 (100)	368,095 ± 29,569 (100)	1,766 ± 405 (100)	63.6 ± 4.0 (100)	82.2 ± 4.1 (100)
Anti-idiotypic	35,253 ± 3,206 (12.03)	412,199 ± 15,959 (111.98)	1,684 ± 327 (95.36)	20.4 ± 2.6 (32.08)	72.7 ± 4.4 (88.56)
Anti-idiotypic serum absorbed with Lyt-1 <sup>+</sup> cells	229,623 ± 26,250 (78.36)	388,846 ± 27,821 (105.64)	1,945 ± 264 (110.14)	24.3 ± 3.0 (38.21)	72.1 ± 6.6 (87.71)
Anti-idiotypic serum absorbed with Lyt-2.3 <sup>+</sup> cells	54,290 ± 15,205 (18.53)	369,143 ± 16,404 (100.28)	1,396 ± 207 (79.05)	53.9 ± 4.0 (84.75)	67.2 ± 5.3 (81.75)

An anti-idiotypic antiserum of C57BL/6 anti-(C57BL/6 anti-CBA) specificity was selected as described in Materials and Methods. The 1:30 diluted antiserum was absorbed three times for 30 min at 4°C with  $1 \times 10^8$  purified C57BL/6 anti-CBA T-cell subpopulations as indicated. Inhibition of MLC and CML was performed as described in Materials and Methods. MLC responses were measured on day 4 and CML on day 6.

C57BL/6 spleen cells were used as responder cells in a MLC against CBA and DBA/2 stimulator cells. Responder cells were treated with either normal C57BL/6 serum or unabsorbed or absorbed anti-idiotypic antiserum and complement (see Materials and Methods).

Cytolytic C57BL/6 T lymphocytes with specificity for CBA and DBA/2 targets were elicited in a MLC and harvested on day 6 of cultures, incubated with normal C57BL/6 serum, absorbed or unabsorbed anti-idiotypic antiserum and complement. Killer cell to target cell ratio was 50:1. 4-h assay.

was thus about 12% of the control response, whereas third-party reactivity against DBA/2 stimulators was hardly touched. The CML response against CBA targets was reduced to about 32% of the control value, but reactivity against DBA/2 was only marginally inhibited (about 88% of the control value). The anti-idiotypic antiserum absorbed with C57BL/6 anti-CBA Lyt-1<sup>+</sup>2<sup>-</sup>3<sup>-</sup> cells showed the "normal" suppressive effect on cytolytic effector cells against CBA (about 38% of the control value) but was no longer an efficient inhibitor of the MLC (78% response of the control value). In both systems, third-party reactivity was left largely unaffected. The same antiserum absorbed with C57BL/6 anti-CBA Lyt-1<sup>-</sup>2<sup>+</sup>3<sup>+</sup> cells could still specifically suppress the MLC response (about 18% of the control) but was now without power to inhibit the CML response. Third-party responses were again not touched.

In summary, an anti-idiotypic antiserum of anti-(C57BL/6 anti-CBA) specificity, capable of inhibiting both MLC and CML in a specific way, would, when absorbed with C57BL/6 anti-CBA Lyt-1<sup>+</sup>2<sup>-</sup>3<sup>-</sup> cells, lose its ability to block the MLC response but could still inhibit CML in a specific way. On the other hand, absorption of the very same antiserum with C57BL/6 anti-CBA Lyt-1<sup>-</sup>2<sup>+</sup>3<sup>+</sup> cells resulted in an antiserum still able to suppress MLC in a specific way but no longer able to inhibit CML responses. Together, these data would strongly suggest that helper and killer T cells generated in the same MLC do indeed carry different groups of idiotypic determinants.

*4. Induction of Specific Cytolytic T Lymphocytes in the Absence of Helper Lyt-1<sup>+</sup>2<sup>-</sup>3<sup>-</sup> Cells.* We have shown previously (11) that autoanti-idiotypic antibodies can be used to induce primary and secondary specific immune responses against alloantigens in the absence of the corresponding antigens. In other words, such autoanti-idiotypic antibodies can functionally mimic the foreign histocompatibility antigens. An antiserum of anti-(C57BL/6 anti-CBA) specificity could thus induce cytolytic T lymphocytes able to eliminate H-2<sup>k</sup> but not H-2<sup>d</sup> targets (11). These experiments were performed with purified T-cell populations. We then asked the question whether it would be possible to induce cytolytic T lymphocytes in the absence of helper Lyt-



TABLE IV  
*Induction of Specific Cytolytic T Lymphocytes by Anti-Idiotypic Antibodies in the Absence of Helper Cells*

Experiment No.	Cells	Incubated with	CBA		CML response			
			100:1	12.5:1	BALB/c		C57BL/6	
					100:1	12.5:1	100:1	12.5:1
I	C57BL/6 T cells	CBA spleen cells	89.4 ± 6.3	70.4 ± 6.7	14.3 ± 2.7	4.5 ± 0.8	-0.7 ± 1.2	1.3 ± 0.4
	C57BL/6 T cells treated with anti-Lyt-1 + C	CBA spleen cells	10.4 ± 4.2	5.8 ± 1.2	4.8 ± 0.7	0.8 ± 0.1	0.3 ± 0.2	2.3 ± 0.5
	C57BL/6 T cells	Anti-idiotypic serum	108.3 ± 5.4	64.2 ± 3.7	5.7 ± 1.2	3.5 ± 1.2	1.6 ± 0.4	0.62 ± 0.2
	CBA T cells	Anti-idiotypic serum	11.4 ± 2.3	9.8 ± 4.2	3.5 ± 1.2	6.7 ± 2.0	0.6 ± 0.4	4.1 ± 1.2
	C57BL/6 T cells treated with anti-Lyt-2.3 + C	Anti-idiotypic serum	0.5 ± 0.1	6.3 ± 0.4	0.64 ± 0.1	0.9 ± 0.1	-1.0 ± 0.1	-0.4 ± 0.3
	C57BL/6 T cells treated with anti-Lyt-1 + C	Anti-idiotypic serum	96.4 ± 10.4	64.0 ± 5.7	11.5 ± 1.2	2.7 ± 0.8	0.0 ± 0.3	-0.79 ± 0.2
	C57BL/6 T cells	CBA spleen cells	79.6 ± 4.8	37.5 ± 3.4	11.2 ± 2.1	0.8 ± 0.2	ND*	ND*
	C57BL/6 T cells treated with anti-Lyt-1 + C	CBA spleen cells	13.5 ± 2.6	1.8 ± 1.0	0.57 ± 0.9	-0.4 ± 1.5	ND	ND
II	C57BL/6 T cells	Anti-idiotypic serum	63.3 ± 6.0	37.8 ± 6.8	10.8 ± 1.3	1.3 ± 1.6	ND	ND
	CBA T cells	Anti-idiotypic serum	0.1 ± 0.7	0.37 ± 1.5	1.1 ± 2.0	0.83 ± 0.3	ND	ND
	C57BL/6 T cells treated with anti-Lyt-1 + C	Anti-idiotypic serum	67.6 ± 4.0	33.6 ± 2.4	2.6 ± 0.6	0.53 ± 1.1	ND	ND
	C57BL/6 T cells treated with anti-Lyt-2.3 + C	Anti-idiotypic serum	-0.13 ± 2.1	-0.03 ± 2.0	2.37 ± 1.3	-1.5 ± 1.7	ND	ND

\* ND, not done.

C57BL/6 cytolytic T lymphocytes were induced in the following ways: (a) with CBA spleen cells (usual procedure, see Materials and Methods), (b) with anti-idiotypic antiserum of C57BL/6 anti-CBA specificity. 15 ml of a cell suspension containing  $3 \times 10^6$  cells/ml was incubated in 3013 Falcon tissue culture flasks in the presence of 0.1% anti-idiotypic antiserum. Cultures were harvested on day 6, and CML responses were measured in a 5-h assay. Effector to target cell ratios were 100:1 and 12.5:1.

$I^+2^-3^-$  T cells. The very same antiserum used to perform the experiments shown in Table III was also tested for its ability to induce specific cytolytic T lymphocytes in vitro. It was found that a serum concentration of 0.1–0.3% was optimal for the induction of H-2<sup>k</sup>-specific C57BL/6 killer cells (data not shown). In another experiment (experiment I in Table IV), a second antiserum of the same specificity has been used which previously has been shown to induce specific primary or secondary immune T-cell responses in the very same system (these latter experiments are given in reference 11).

Normal C57BL/6 T lymphocytes or their Lyt subpopulations were incubated with either irradiated CBA spleen cells as stimulating antigens or with anti-idiotypic antibodies of anti-(C57BL/6 anti-CBA) specificity. Cultures were harvested on day 6, and the surviving lymphocytes tested for their ability to lyse H-2<sup>k</sup>, H-2<sup>d</sup>, or H-2<sup>b</sup> targets in a <sup>51</sup>Cr release assay. The restricted availability of anti-idiotypic antisera did not allow parallel measures of cellular proliferation. The results are shown in Table IV. In the two experiments, CBA spleen cells, as well as anti-idiotypic antibodies, induced specific killer T lymphocytes capable of lysing CBA but not BALB/c targets. The same anti-idiotypic antisera had no effect on CBA T cells. When C57BL/6 T lymphocytes purified via treatment with anti-Lyt-1 serum and complement were used, there was a failure of CBA stimulator cells to induce cytolytic T cells. However, anti-idiotypic antisera were still fully able of inducing highly specific killer T lymphocytes in the same purified Lyt-1<sup>-</sup>2<sup>+</sup>3<sup>+</sup> cell population. Treatment of C57BL/6 T

cells with anti-Lyt-2.3 serum and complement (elimination of the killer precursors) resulted in a T-cell population that was no longer able to differentiate into specific cytolytic T cells regardless of stimulus. From the above data we would thus conclude that autoanti-idiotypic antibodies can serve as a stronger stimulus for the induction of anti-H-2 specific killer T cells than the actual potential targets themselves.

### Discussion

The present results provide information of T-cell specificity and regulation at several levels. Firstly, killer T cells are known to be able to function as single effector cells in an autonomous manner during the lytic effector phase (17). The present results, showing the specific elimination of cytolytic ability by treating effector killer T cells in vitro with anti-idiotypic antibodies and complement, prove the actual presence of idiotypic receptors on such T cells. The conclusion that killer T cells are idiotype positive had previously only been inferred from indirect evidence (11).

Secondly, the present results provide new knowledge of the distribution pattern of idiotypes on T cells with different Lyt phenotypes. Data from two different approaches, induction of specific unresponsiveness by autoanti-idiotypic immunity and absorption of anti-idiotypic antibodies by different Lyt blasts generated in MLC, strongly indicate that  $\text{Lyt-1}^+\text{2}^-\text{3}^-$  and  $\text{1}^-\text{2}^+\text{3}^+$  blasts generated in the same MLC across an H-2 barrier express distinct groups of idiotypes. This accords with the common view that the dominating, proliferating T blast in conventional MLC is reactive against allo-Ia antigens, whereas the major killer T-cell specificity is directed against H-2K/D alloantigens (1, 2, 18). The former T blasts would mainly express the  $\text{Lyt-1}^+\text{2}^-\text{3}^-$  phenotype, whereas the killer T cells in allogeneic combinations normally are of  $\text{Lyt-1}^-\text{2}^+\text{3}^+$  (1). That the two subgroups of MHC-reactive T blasts defined by Lyt phenotype exhibit differential antigen-binding specificity has been indicated in systems where actual selective binding of allo-Ia antigens to  $\text{Lyt-1}^+\text{2}^-$  blasts and to H-2 K/D molecules was demonstrated (19) to the responding  $\text{Lyt-1}^-\text{2}^+$  cells.

The present results further confirm the dichotomy of antigen-binding specificity between these two subgroups of T cells and suggest that this difference, at least in the alloantigenic systems studied, may be of an almost absolute nature. It is possible, however, that this striking difference is secondary to proliferative selection according to Lyt phenotype and specificity during MLC, and that differential expression of idiotype profiles at the virgin T-cell level may be less restricted. It should also be noted that the present results should not necessarily be taken as evidence that there will always exist such a clear cut idiotypic dichotomy between immune  $\text{Lyt-1}^+\text{2}^-\text{3}^-$  and  $\text{Lyt-1}^-\text{2}^+\text{3}^+$  T cells, but that this may be confined to systems where T-cell subgroups clearly display different restrictions as to MHC of Ia or H-2K/D structures. There are as yet no data indicating whether helper and suppressor T cells with different Lyt phenotypes but with specificity for the same epitope on a soluble antigen may express the same or a similar idiotype (9, 10). Finally, the present study indicates that autoanti-idiotypic antibodies may display a striking "helping" power in the induction of specific cytolytic killer T cells in the absence of alloantigenic stimulator cells. We have previously shown this to be possible with normal splenic T cells in vitro (10, 11), but we show here (Table IV) that autoanti-idiotypic antibodies can exert the same function on pure, normal  $\text{Lyt-1}^-\text{2}^+\text{3}^+$  T lymphocytes. This is explicable from

the viewpoint that such a population is known to constitute the major precursor cell pool for alloreactive killer T cells (1). But the result is surprising in the sense that such a successful induction of cytolytic cells normally requires  $\text{Lyt-1}^+2^-3^-$  cells functioning as helpers. This is also confirmed in Table IV where allogeneic cells used as stimulating agents could only induce killer T-cell formation when both  $\text{Lyt-1}^+2^-3^-$  and  $\text{Lyt-1}^-2^+3^+$  cells were present among the responder T cells. The present data nevertheless do not necessarily mean that the anti-idiotypic antibodies can fully replace conventional helper T cells (they could be extremely efficient triggers of a few  $\text{Lyt-1}^+2^-3^-$  cells that may have survived the anti-Lyt treatment); but there is the intriguing possibility that anti-idiotypic helping reactions may, in fact, be a normal feature of MLC-helper T-cell function. It is already known, from several other systems, that participation of anti-idiotypic-specific helper cells may be highly important in the generation of idiotypic-positive antibody (9, 20-22). Experiments to explore this possibility with regard to MLC helper activity are under way.

In conclusion, the present results show that both proliferating and cytolytic T cells generated in MLC express idiotypic receptors, and that the spectra of idiotypes displayed is unique for each subgroup defined by Lyt phenotype and function. Autoanti-idiotypic antibodies are shown capable of efficient induction of specific cytolytic T cells from a population comprising normal  $\text{Lyt-1}^-2^+3^+$  cells. The latter finding may suggest that helper T cells may function in the generation of cytolytic T cells, at least in part, through the medium of autoanti-idiotypic reactions.

### Summary

Killer T cells with specificity for major histocompatibility antigens have been shown in mice and rats to display idiotypic receptors allowing the lysis of such cells at the effector phase by anti-idiotypic antibodies and complement. A comparison was made between idiotypes displayed by  $\text{Lyt-1}^-2^+3^+$  and  $\text{Lyt-1}^+2^-3^-$  T blasts, generated in the same mixed leucocyte culture (MLC), across an entire H-2 locus barrier. This was done by absorption of anti-idiotypic antibodies with respective T blasts, followed by estimation of the ability of the absorbed antiserum to inhibit MLC or killer T-cell function. Further, the capacity of Lyt-purified, MLC-generated T blasts to provoke specific unresponsiveness via anti-idiotypic immunity in syngeneic recipients was analyzed. Taken together, the results demonstrate that  $\text{Lyt-1}^+2^-3^-$  T blasts responsible for the major part of MLC proliferation have distinctly different idiotypes from those on the  $\text{Lyt-1}^-2^+3^+$  killer T cells. That the idiotypes on the killer T-cell precursors can serve as triggering sites for induction of effector T-cell function was then suggested by experiments with  $\text{Lyt-1}^-2^+3^+$ -purified, normal T cells as precursor cells in vitro. The fact that autoanti-idiotypic antibodies may circumvent the need for helper  $\text{Lyt-1}^+2^-3^-$  T cells in the generation of allospecific killer T cells indicates that the former cells may normally function partly via such anti-idiotypic reactions.

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