

T CELL RECEPTORS WITH ALLO-MAJOR  
HISTOCOMPATIBILITY COMPLEX  
SPECIFICITY FROM RAT AND MOUSE\*  
Similarity of Size, Plasmin Susceptibility, and  
Localization of Antigen-binding Region

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T cells and B cells can recognize antigenic determinants via antigen-specific receptors (1-4). Such receptors on mouse B cells are basically conventional immunoglobulins coded for by genes located on chromosomes 12 (heavy chain), 6 (kappa chain), and 9, 13, or 16 (lambda chain) (5-7). The antigen-specific receptor on T cells is considered elusive (8). The mystery of this receptor has been elucidated by the finding that anti-idiotypic antisera react both with specific antibodies and with specific T cells (9, 10), and that the idiotypes are localized on antigen-specific receptors on T lymphocytes. Genetic studies showed that T cell idiotypes were coded for by genes linked to the heavy chain constant region genes (11, 12). As we failed to demonstrate either conventional light chain or heavy chain determinants, we suggested that T cell receptor molecules were built up of a variable region coded for by genes located on chromosome 12 (mouse) and a private T cell constant region. This T cell heavy chain we called Tau-chain (13). However, precise data on the structure of the T cell receptor are not abundant. Several different types of molecules have been described, thought to represent either antigen-specific T cell receptor molecules or molecules that communicate within the subsets of the immune cell orchestra (14-27). Primary characteristics of all molecules described so far include the lack of conventional immunoglobulin C<sub>H</sub>, V<sub>L</sub>, or C<sub>L</sub> determinants, and the presence of V<sub>H</sub> determinants (mainly idiotypes) within a heavy chain. Some of the reported T cell-derived molecules in addition carry determinants coded for by genes of the major histocompatibility complex (MHC)<sup>1</sup> of the species, indicating that products of the genes of chromosome 12 are associated with products of genes of chromosome 17 (mouse) (19).

Our previously published experiments (14) on alloreactive, idiotypic T cell receptors from normal or specifically activated T cells showed monomeric or dimeric polypep-

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<sup>1</sup> *Abbreviations used in this paper:* CNBr, cyanogen bromide; Con A, concanavalin A; D-PBS, Dulbecco's modified phosphate-buffered saline; EHAA, Eagle's high amino acid; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; MHC, major histocompatibility complex; MLC, mixed leukocyte culture; NRS, normal rabbit serum; PHA, phytohemagglutinin; SDS, sodium dodecyl sulfate; TCGF, T cell growth factor.

tide chains of ~70,000 daltons carrying neither Ig determinants of heavy or light chain type nor determinants coded for by the MHC. Extraction of the T cell receptor molecules were carried out with anti-idiotypic antibodies. Such antibodies gave us information about V<sub>H</sub> gene products on a subset of the alloreactive T cell pool, but they were useless for an analysis of constant regions of the T cell receptors. We therefore tried to obtain antibodies directed at the constant region of T cell receptors by using purified idiotypic, alloreactive T cell receptors as an immunogen. Prolonged immunization and absorption yielded rabbit antisera reacting with C<sub>r</sub> of T cell receptors from both rats and mice present on normal as well as on activated T cells of both helper and effector cell type, but not on B cells. The same antiserum induced proliferative T cell responses. Using this antiserum we were also able to isolate T cell receptor molecules capable of recognizing antigenic determinants. This molecule consists of a polypeptide chain of ~70,000 daltons that can be split by plasmin and acid into two main peptides of ~45,000 and 25,000 daltons. The smaller of the two peptides appears to carry the antigen-binding capacity.

### Materials and Methods

*Animals.* Mice of the inbred strains CBA/J (H-2<sup>b</sup>), DBA/2J (H-2<sup>d</sup>), and C57BL/6J (H-2<sup>b</sup>) were purchased from Bomholtgard Ltd., Ry, Denmark, and maintained in our own colony. Mice were ~8 wk of age when used for the experiments. Rats of the inbred strains Lewis (RT<sup>e</sup>), DA (RT1<sup>a</sup>), August (RT1<sup>c</sup>), AVN (RT1<sup>a</sup>), BN (RT1<sup>b</sup>), and L.BN (RT1<sup>b</sup>) were bred and maintained in our own colony (28). Animals were ~10 wk of age when used for experiments. Sex-matched mice or rats were used within each experiment.

*Preparation of Lymphoid Cell Suspensions.* Spleens and cervical lymph nodes were aseptically removed and single cell suspensions were prepared by pressing through a stainless steel mesh in Dulbecco's modified phosphate-buffered saline (D-PBS) (Ca<sup>++</sup> and Mg<sup>++</sup> free) medium. Large particles were removed by sedimentation and single cell suspensions were washed once in D-PBS. Erythrocytes were lysed by hypotonic shock, 0.9 ml of distilled water was added to the cell pellet of five mouse spleens or two rat spleens for 2 s followed by 0.1 ml of 10 times concentrated D-PBS and 10 ml of isotonic D-PBS. The lymphoid cell suspension was sedimented at 400 g and resuspended either in D-PBS, or, when intended for use in mixed leukocyte culture (MLC), in Eagle's high amino acid (EHAA) medium. The cell number was determined with a Biophysics 6300 A Cytograph (Biophysics Systems Inc., Mahopac, N.Y.).

*Preparation of T Lymphocytes.* B lymphocytes were removed on Ig-anti-Ig columns (29) of relevant nature using controlled pore glass beads, 80-120 mesh (Serva, Heidelberg, West Germany).

Lymphoid cell suspensions at concentrations of  $<5 \times 10^7$  cells/ml D-PBS were passed over the doubly coated glass beads with a flow rate of 30 drops/min. Cells passing the columns were harvested and washed once in D-PBS. The preparations contained <1% B lymphocytes when tested with a fluorescein isothiocyanate (FITC)-labeled rabbit anti-rat Ig or rabbit anti-mouse Ig.

*MLC.* Cells were prepared according to the procedure described above. MLC were performed in flat bottomed microtiter plates (Cook M220-20 ART, Greiner Ltd., Nürtingen, West Germany) using  $0.25 \times 10^6$  responder lymphocytes and  $0.5 \times 10^6$  2,500 rad irradiated stimulator cells. EHAA medium was complemented with  $5 \times 10^{-6}$  M 2-mercaptoethanol (2-ME) and 0.5% fresh mouse (pooled from responder and stimulator strain mice) or 1% fresh serum from normal BN rats, respectively.

Cultures were pulsed for 6 h with 1  $\mu$ Ci of [<sup>3</sup>H]TdR (specific activity 40-60 Ci/mmol, New England Nuclear, Boston, Mass.) before the harvesting time as indicated in the figures. Cultures were harvested using a Skatron multicell harvester (Skatron AS, Lierbyen, Norway). Glass fiber filters were counted in 2 ml of scintillation fluid in a liquid scintillation counter (Mark III, model 6880, Searl Radiographics Inc., Des Plaines, Ill.).

*Protein A Assay.* This assay was performed as described previously (30) in round-bottomed

microtiter plates (Cook M24-AR Greiner Ltd.). Cells were transferred into counting tubes and counted in an Intertechnique Gamma Counter (model CG 4000), equipped with a 3-in. well-type crystal.

*Preparation of T Cell Growth Factor (TCGF; Concanavalin A Supernate).* Rat lymphocytes were cultured in EHAA medium complemented with 0.5% fresh normal BN rat serum and stimulated with 5  $\mu\text{g/ml}$  of concanavalin A (Con A, Pharmacia Fine Chemicals, Uppsala, Sweden). 40 ml of a suspension containing  $5 \times 10^6$  lymphocytes/ml was cultured in a 3024 Falcon tissue culture flask and the supernate harvested 48 h later, sterile filtered, and kept at  $-20^\circ\text{C}$ . Con A was neutralized by adding  $\alpha$ -methylmannoside.

*Expansion of Rat MLC T Lymphoblasts with TCGF.* Primary MLC T lymphoblasts were prepared using the conditions described above. On day 8 of culture primed T lymphocytes were restimulated with the corresponding stimulator cells using the same conditions as described for the primary MLC. 1 wk later living cells were harvested and purified on Ficoll-Paque (31). These cells were expanded on  $F_1$  macrophages and TCGF in the following way. Macrophages were harvested from the peritoneal cavity of  $F_1$  rats, washed once, and irradiated with 2,500 rad. Macrophages obtained from one rat were distributed over five 3013 Falcon tissue culture flasks and cultured in 5 ml EHAA medium, complemented with 10% fetal calf serum (FCS) and 20% TCGF for 24 h. 10 ml of a suspension containing  $1.25 \times 10^6$  restimulated MLC T lymphocytes (see above) in EHAA medium complemented with 10% FCS and 20% TCGF was added to each flask. Lewis anti-DA MLC T cells were cultured on (Lewis  $\times$  DA) $F_1$  macrophages and Lewis anti-BN MLC T cells on (Lewis  $\times$  BN) $F_1$  macrophages, respectively. Cells were harvested 1 wk later and cultured on new macrophages under the same conditions by dividing the harvested cells from 1 flask into 10 new 3013 flasks. The procedure was repeated four times. Cells were finally harvested 3 d after the last transfer, purified on Ficoll-Paque, and used for the experiments. During each weekly transfer procedure part of the blasts were used for experimental purposes.

*Stimulation of Rat Lymphocytes with Mitogens.* Rat lymphocytes were prepared as described above. 15 ml of a suspension containing  $3 \times 10^6$  cells/ml EHAA medium complemented with 0.5% fresh normal BN serum were added per 3013 Falcon tissue culture flask with mitogens of the following concentrations: Con A, 3  $\mu\text{g/ml}$ ; phytohemagglutinin (PHA) (Wellcome, purified PHA, Wellcome Research Laboratories, Beckenham, England), 1.5  $\mu\text{g/ml}$ ; lipopolysaccharide (LPS, *Escherichia coli* 055:B5 from Difco Laboratories, Detroit, Mich.), 10  $\mu\text{g/ml}$ . Cells were harvested on day 3 of culture and purified on Ficoll-Paque (31).

*Fluorescence Technique.*  $3 \times 10^6$  cells as stated in the tables were incubated with 100  $\mu\text{l}$  of 1:20 diluted antiserum 801 (see below) or normal serum for 1 h at  $4^\circ\text{C}$ . After three washes cells were incubated with 100  $\mu\text{l}$  1:20 diluted FITC-labeled  $F(\text{ab})_2$  of sheep anti-rabbit IgG for 1 h at  $4^\circ\text{C}$ . Cells were washed with ice-cold D-PBS containing 10% FCS and 10 mM  $\text{NaN}_3$ . Fluorescence was examined with a Zeiss standard 18 microscope (Carl Zeiss, Göttingen, Germany) by incident light illumination.

*Stimulation of T Lymphocytes with Rabbit Anti-Rat T Lymphocyte Receptor Serum 801.* Rat or mouse T lymphocytes were prepared from spleens and lymph nodes as described above. T cells were stimulated with rabbit antiserum 801 either in flat-bottomed microtiter plates or 3013 Falcon tissue culture flasks (Falcon Labware, Oxnard, Calif.) by using EHAA medium complemented with  $5 \times 10^{-5}$  M 2-ME and 0.5% fresh normal BN serum. Serum 801 or normal rabbit serum was used at 0.5% concentration. For cultures in microtiter plates 3 or  $5 \times 10^5$  T lymphocytes in 200  $\mu\text{l}$  medium were used per well. Each 3013 flask contained 15 ml of a suspension containing  $1.25 \times 10^6$  cells/ml. Stimulation was measured by [ $^3\text{H}$ ]TdR incorporation as described under MLC.

*Preparation of Rabbit Anti-Rat T Lymphocyte Receptor Antiserum (Serum 801).* Rat T cell receptor material was isolated from normal Lewis serum and Lewis anti-DA MLC supernate by absorbing normal Lewis serum and MLC supernate on an anti-(Lewis anti-DA) anti-idiotypic immunosorbent. The anti-idiotypic nature of the antiserum pool 3 of specificity anti-(Lewis anti-DA) has been published in detail (32). The Ig fraction of this antiserum was coupled to cyanogen bromide (CNBr)-activated Sepharose 4B following the instructions of the manufacturers (Pharmacia Fine Chemicals). Material was absorbed on the anti-idiotypic immunosorbent and eluted by glycine-HCl buffer, pH 2.8, containing 2 M NaCl followed by 3 M  $\text{MgCl}_2$ ,

dialyzed against D-PBS and concentrated by negative pressure dialysis. Four rabbits were immunized with 50  $\mu$ g of eluted protein emulsified in Freund's complete adjuvant for the first injection and incomplete for the boosters. The immunogen was applied subcutaneously at several sites over the back. Rabbits were immunized 12 times at  $\sim$ 1 mo intervals. The first bleeding was taken 10 d after the fourth injection and subsequently 10 d after each further injection. Sera was absorbed sequentially on Sepharose immunosorbents on which nude mouse serum, nude rat serum, rat Ig, rat serum albumin, mouse serum albumin, or FCS were coupled. Sera were sterile filtered and kept at  $-20^{\circ}\text{C}$ .

*Purification of Internally Labeled Receptor Molecules with Antiserum 801 and External Labeling Procedure of Purified T Cell Receptor Material.* MLC T lymphoblasts obtained and expanded as described above were purified on Ficoll-Paque (31).  $2 \times 10^7$  purified T blasts in 10 ml of EHAA medium were internally labeled in 3013 Falcon tissue culture flasks with 0.3 mCi of  $^3\text{H}$ -amino acid mixture (NET-250, New England Nuclear), 0.3 mCi of [ $^3\text{H}$ ]leucine (TRK.636, The Radiochemical Centre, Amersham, England) and 0.3 mCi of [ $^{35}\text{S}$ ]methionine (NEG-009T; New England Nuclear) for 48–72 h. Cells were collected, centrifuged for 6 min at  $1,500 \times g$ , and the supernate, to which phenylmethylsulphonyl fluoride (1 mM),  $\epsilon$ -aminocaproic acid (0.1%), 0.1 mM tosyllysinechloromethylketone, and 1 mM EDTA were added, was absorbed on an 801-immunosorbent.

This immunosorbent was prepared from absorbed antiserum 801 by coupling the IgG fraction (DEAE-purified [9]) onto CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals). 100 ml internally labeled supernate was absorbed three times on a  $1 \times 5$ -cm immunosorbent with a flow rate of 30 ml/h. The column was extensively washed for 4 h or overnight with 500 ml D-PBS at  $4^{\circ}\text{C}$ . The bound material was eluted with 3 M KSCN. Eluted material was dialyzed against D-PBS, concentrated in the dialysis bag by the use of cane sugar, dialyzed against D-PBS, absorbed on Con A-Sepharose immunosorbent, concentrated again as above, and stored at  $-70^{\circ}\text{C}$ . Several different preparations were pooled.

For external labeling procedures receptor material was isolated as described above. 50  $\mu$ g of eluted protein in 100  $\mu$ l D-PBS was iodinated with  $^{125}\text{I}$  (Eidgenössisches Institut für Reaktorforschung, Würenlingen, Switzerland) for 1 min at room temperature by adding 25  $\mu$ l of chloramin T (1 mg/ml) followed by 25  $\mu$ l of sodium metabisulfite (1 mg/ml). Free iodine was removed by filtration of the iodinated material through a Sephadex G-25 column.

*Digestion of T Cell Receptor Material with Plasmin.* Material to be digested was purified from MLC supernate as describe above. 1 ml of purified material (internally or externally labeled) in D-PBS corresponding to about 5  $\mu$ g of protein was adjusted to pH 2.5 with 1 N HCl and incubated for 60 min at room temperature. The solution was adjusted to neutral pH with 1 N NaOH, and 25  $\mu$ l of plasmin (Sigma Chemical Co., St. Louis, Mo.; 0.1  $\mu$ g/ml) was added and the mixture was incubated for 30 min at room temperature. The resulting products were analyzed on SDS-gels or separated on G-200 Sephadex in 6 M urea and 0.1 M sodium acetate, pH 4.5. Radioactivity was determined and the corresponding peaks were pooled, dialyzed, concentrated in the dialysis bag with cane sugar, and dialyzed against PBS.

*Polyacrylamide Gel Electrophoresis.* Electrophoresis was carried out in 10-cm plastic tubes or slab-gels on 10% (wt/vol) acrylamide essentially as described elsewhere (33). For internally labeled material electrophoresis was carried out in gels containing *N,N'*-diallyl-tartardiamine instead of *N,N'*-methylenebisacrylamide as described elsewhere (34). 1-mm slices were dissolved overnight in 0.3 ml of 2% periodic acid and counted in 2 ml of Biofluor (New England Nuclear) in plastic minivials in a Mark III scintillation system (model 6880, Searle Radiographics Inc., Des Plaines, Ill.).

*Treatment of Mouse T Lymphocytes with Anti-Lyt Antisera.* Anti-Lyt 1.2, 2.2, and 3.2 were a generous gift of Dr. Fung W. Shen, Memorial Sloan-Kettering Cancer Center, New York; others were produced in Uppsala, Sweden, as described (35) or purchased from New England Nuclear. 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 lymphocytes 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 then washed three times and used for the experiments.

## Results

*Binding of Rabbit Anti-Rat T Cell Receptor Antiserum onto Normal Rat and Mouse T Lymphocytes As Demonstrated by the Protein A Assay.* In a first series of experiments we tested whether different rabbit antisera elicited by immunization with rat T cell receptor material (absorbed and eluted from anti-idiotypic immunosorbent) would bind onto rat and mouse normal T lymphocytes. Such T cells were prepared from spleens and lymph nodes from Lewis, DA, and BN rats as well as from C57BL/6, CBA, and DBA/2 mice as described in Material and Methods. T lymphocytes were incubated with different dilutions of rabbit antisera, washed, and incubated with  $^{125}\text{I}$ -labeled protein A. No significant binding occurred with any of the four rabbit sera up to the seventh immunization. Fig. 1 demonstrates data of rabbit serum 801 which represents a pool of the bleedings of one rabbit from the 9th to the 12th immunization. This pool was extensively absorbed with different immunosorbents (see Materials and Methods) and showed no reaction with serum components as assessed by precipitation or radioimmune tests. The data demonstrate that serum 801 contained antibodies of IgG class able to recognize surface structures of rat and mouse T lymphocytes up to dilutions of 1:243. The binding of antiserum 801 onto mouse T lymphocytes was somewhat weaker when compared with rat T lymphocytes, but was still highly significant. The other three rabbit antisera tested contained antibodies capable of significant binding onto rat and mouse T lymphocytes, but had either lower titers or high titers of antibodies reacting with non-T lymphocytes. We therefore decided to continue for the following experiments to use antiserum 801.

*Binding of Rabbit Anti-Rat T Cell Receptor Antiserum onto Normal Rat and Mouse T Lymphocytes as Demonstrated by Indirect Immunofluorescence.* Using the protein A assay we obtained information indicating that rabbit antiserum 801 contains IgG antibodies capable of reaction with rat and mouse T lymphocytes. We wanted to know with what percentage of rat or mouse T lymphocytes this antiserum would react. We therefore tested antiserum 801 by the indirect immunofluorescence technique. Lewis,

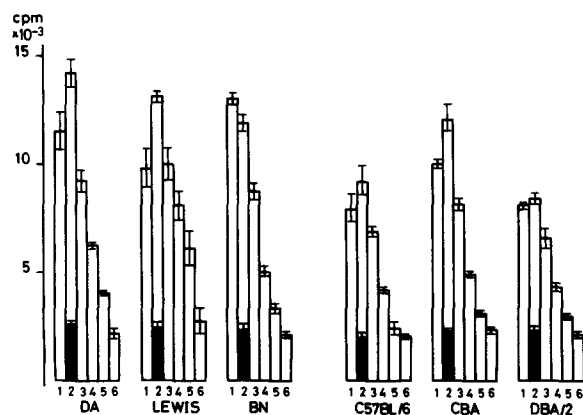


FIG. 1.  $3 \times 10^6$  rat or mouse T lymphocytes (prepared as described in Materials and Methods) were incubated with antiserum 801 for 1 h at  $4^\circ\text{C}$ , washed and incubated with antiserum 801 for 1 h at  $4^\circ\text{C}$ , washed and incubated with 50,000 cpm of  $^{125}\text{I}$ -labeled protein A. Serum dilutions: 1 = 1:3, 2 = 1:9, 3 = 1:27, 4 = 1:81, 5 = 1:243 and 6 = 1:729. Columns represent mean cpm of duplicates  $\pm$  SE. Three different NRS were tested at a dilution of 1:9, and the means of these tests are indicated as dashed columns.

TABLE I  
*Binding of Antiserum 801 onto Rat and Mouse Normal T Lymphocytes As  
 Demonstrated by Indirect Immunofluorescence*

Cells	Serum	Percent positive cells
Lewis T	801	77.1
Lewis T	NRS	1.0
DA T	801	78.9
DA T	NRS	1.7
BN T	801	68.3
BN T	NRS	1.1
C57BL/6 T	801	77.0
C57BL/6 T	NRS	1.6
CBA T	801	69.9
CBA T	NRS	2.0
DBA/2 T	801	72.1
DBA/2 T	NRS	1.9

Rat and mouse T lymphocytes were prepared as described in Materials and Methods.  $3 \times 10^6$  T cells were incubated with 100  $\mu$ l of 1:20 diluted antiserum 801 or NRS for 1 h at 4°C using D-PBS with 10% FCS and 10 mM NaN<sub>3</sub>. After washing, cells were incubated for 1 h at 4°C with a 1:20 dilution of FITC-labeled F(ab')<sub>2</sub> of sheep anti-rabbit IgG.

DA, and BN rat T lymphocytes and C57BL/6, CBA, and DBA/2 mouse T lymphocytes were incubated with antiserum 801 followed by FITC-labeled F(ab')<sub>2</sub> fragments of sheep anti-rabbit IgG. The test was carried out under noncapping conditions (see Materials and Methods). As listed in Table I ~75% of rat or mouse T lymphocytes showed typical membrane fluorescence with serum dilutions giving plateau levels. Two populations of positive cells could be identified, one with bright fluorescence and one with rather weak and dull, but still significant fluorescence when compared with the fluorescence caused by normal rabbit serum (NRS). Approximately 20–30% of rat or mouse T lymphocytes were completely negative. The intensity of fluorescence of positive cells in both rat and mouse T lymphocytes was about the same. Under the same conditions mouse T lymphocytes were not stained with NRS.

*Binding of Rabbit Antiserum 801 onto Different T Lymphoblasts and T Cell Subpopulations but Not to B Blasts as Assessed by Protein A Assay or Indirect Immunofluorescence.* The results obtained to this point had shown the selective reactivity of antiserum 801 with a majority but not all of normal lymphocytes in mice and rats. The next question we asked was whether the antiserum would also react with T or B lymphoblasts induced either by different mitogens or by allogeneic stimulator cells and with both Lyt-1<sup>+</sup>,2<sup>-</sup>,3<sup>-</sup> and Lyt-1<sup>-</sup>,2<sup>+</sup>,3<sup>+</sup> mouse T cell subpopulations. MLC T lymphoblasts were obtained by restimulation of primary MLC and expansion of the responder cells with TCGF; nonspecific lymphoblasts were induced by either T or B cell mitogens (see Materials and Methods). Blasts were purified on Ficoll-Paque and incubated with antiserum 801 or NRS followed by FITC-labeled F(ab')<sub>2</sub> fragments of sheep anti-rabbit IgG. Table II summarizes a representative experiment. Approximately 80–85% of either rat or mouse MLC T lymphoblasts could be stained with rabbit antiserum 801. Background staining using NRS gave figures approaching 10% indicating a background with blasts about five times higher as compared with normal rat and mouse T lymphocytes. Again, two positive populations with bright or weak fluores-

TABLE II  
*Binding of Antiserum 801 onto Different Lymphoblasts As Demonstrated by Indirect Immunofluorescence*

Cells	Serum	Percent positive cells
Lewis anti-DA MLC T	801	86.2
Lewis anti-DA MLC T	NRS	10.1
Lewis anti-BN MLC T	801	87.2
Lewis anti-BN MLC T	NRS	11.1
C57BL/6 anti-CBA MLC T	801	90.8
C57BL/6 anti-CBA MLC T	NRS	9.1
C57BL/6 Con A	801	62.9
C57BL/6 Con A	NRS	13.8
C57BL/6 LPS	801	16.3
C57BL/6 LPS	NRS	17.2
C57BL/6 PHA	801	23.9
C57BL/6 PHA	NRS	7.6

Primary MLC T lymphoblasts were restimulated on day 8 of culture and expanded with TCGF four times at weekly intervals. Con A, LPS, and PHA blasts were harvested on day 3 of culture (see Materials and Methods). Blasts were purified on Ficoll-Paque. Conditions were otherwise as in the legend of Table I.

TABLE III  
*Binding of Antiserum 801 onto T Cell Subpopulations As Demonstrated by Protein A Assay*

Cells	Serum	Binding of <sup>125</sup> I-labeled protein A (Mean ± SE cpm of triplicates)
C57BL/6 anti-CBA MLC T lymphoblasts	801	6,946 ± 554
	NRS	2,020 ± 319
C57BL/6 anti-CBA MLC T lymphoblasts treated with anti-Lyt-1 and C	801	6,702 ± 798
	NRS	2,372 ± 337
C57BL/6 anti-CBA MLC T lymphoblasts treated with anti-Lyt-2,3 and C	801	3,845 ± 324
	NRS	1,944 ± 313

MLC T lymphoblasts and subpopulations were prepared as described in Materials and Methods.  $1 \times 10^6$  cells were incubated for 1 h at 4°C with antiserum 801 or NRS, 1:20 final dilution, washed and incubated for another hour at 4°C with 50,000 cpm of <sup>125</sup>I-labeled protein A.

cence could be observed as already noted with normal T lymphocytes. Although highly significant, the percentage of positive cells within the mitogen-activated T blasts was lower compared with the MLC T lymphoblasts, with ~60% of Con A blasts

TABLE IV  
*Binding of Antiserum 801 onto Different T Cell Subpopulations As Demonstrated by Indirect Immunofluorescence*

Cells	Serum	Percent positive cells
C57BL/6 anti-CBA MLC T lymphoblasts	801	83.0
	NRS	8.9
C57BL/6 anti-CBA MLC T lymphoblasts treated with anti-Lyt 1 and C	801	73.1
	NRS	12.4
C57BL/6 anti-CBA MLC T lymphoblasts treated with anti-Lyt 2,3 and C	801	68.1
	NRS	6.4

MLC T lymphoblasts and subpopulations were prepared as described in Materials and Methods. Conditions for the fluorescence analysis are the same as described in the legend to Table I.

and 20% of PHA blasts being positive. Using B lymphoblasts induced with LPS, no significant staining could be observed with antiserum 801 compared with NRS, which caused a background staining of ~17%. Thus, rabbit antiserum 801 reacts very strongly with MLC T lymphoblasts of both rats and mice, and to a lower degree with T blasts induced by Con A or PHA. However, the very same antiserum failed to react with B lymphoblasts induced by LPS.

Mouse MLC T lymphoblasts obtained as described above were incubated with anti-Lyt antisera and complement (see Materials and Methods). Purified subgroups of cells were incubated in a first series of experiments with antiserum 801 followed by  $^{125}\text{I}$ -labeled protein A. Table III illustrates such an experiment. Nonseparated MLC T lymphoblasts and  $\text{Lyt-1}^-,2^+,3^+$  cells fixed approximately equal amounts per unit cell number of radiolabeled protein A.  $\text{Lyt-1}^+,2^-,3^-$  cells bound lower, but significant amounts of radioactivity when compared with unseparated or  $\text{Lyt-1}^-,2^+,3^+$  blasts. All three types of cells absorbed roughly the same background amount of radioactivity when incubated with NRS. In a second series of experiments T cell subpopulations prepared as described above were incubated with rabbit antiserum 801 or NRS followed by FITC-labeled  $\text{F(ab')}_2$  fragment of sheep anti-rabbit IgG. Table IV depicts a representative experiment. About 80% of nonseparated vs. 70% of  $\text{Lyt-1}^-,2^+,3^+$  and  $\text{Lyt-1}^+,2^-,3^-$  blasts were stained by antiserum 801. The intensity of fluorescence of  $\text{Lyt-1}^-,2^+,3^+$  cells as judged by eye was significantly brighter than that of  $\text{Lyt-1}^+,2^-,3^-$  cells.

These latter data can be summarized as follows. Antiserum 801 recognizes surface structures of specifically MLC T lymphoblasts and to a certain degree also of nonspecifically induced T lymphoblasts, but fails to react with B lymphoblasts. Both  $\text{Lyt-1}^+,2^-,3^-$  and  $\text{Lyt-1}^-,2^+,3^+$  blasts can be shown to be recognized by antiserum 801, but  $\text{Lyt-1}^-,2^+,3^+$  blasts carry more of the relevant epitopes per cell than the  $\text{Lyt-1}^+,2^-,3^-$  blasts as judged by intensity of the fluorescence or uptake of  $^{125}\text{I}$ -protein A.

*Isolation and Characterization of T Cell Receptor Molecules Released from MLC-activated T Lymphoblasts by the Use of Antiserum 801.* We next analyzed the structures located on the surface of the T lymphocytes recognized by antiserum 801. We have found that when analyzing these structures, the most efficient way to quantify the data is to use



supernates from specifically activated T lymphoblasts from rats or mice. Membrane extraction by the use of antiserum 801 and *Staphylococcus aureus* bearing protein A gave similar results on isolation of molecules but the amount of material and the reproducibility of the experiments favored the use of supernates from specifically activated T lymphoblasts. Such fluid could be absorbed and eluted from an immunosorbent, made out of the IgG fraction of rabbit antiserum 801. The material to be analyzed was either internally labeled with a mixture of tritiated aminoacids and [<sup>35</sup>S]methionine or alternatively was radiolabeled with <sup>125</sup>I after purification. Analysis was performed on sodium dodecyl sulfate (SDS) gels as described in Materials and Methods.

Fig. 2 demonstrates SDS-gel patterns of rat (A) and mouse (B) T cell receptor material isolated from Lewis anti-DA or C57BL/6 anti-CBA MLC supernates by the use of 801 immunosorbent. A main peak was found at ~70,000 daltons in both rat- and mouse-derived material in agreement with previous studies on idiotypic T cell receptors (14). A small shoulder was sometimes present at ~60,000 daltons. In some experiments additional peaks were found near 50,000, 43,000, and 25,000 daltons. We have previously found that these peaks represent degradation products from the 70,000-dalton chain (14). The peak at ~70,000 daltons represents single polypeptide chains that cannot be split into smaller units using strong reducing conditions such as 1 M 2-ME or 1 M dithiothreitol confirming earlier studies (14). The single polypeptide chains carried no detectable constant Ig determinants and no antigenic structures coded for by the MHC of the species, as determined by different absorption studies (see Table V). All this is in perfect consonance with the features of T receptor molecules isolated by anti-idiotypic immunosorbents (13, 14).

Material representing 70,000-dalton molecules eluted from anti-idiotypic immunosorbent and exposed to proteolysis with pepsin or papain were degraded into a variety of defined peptides with different molecular weights (36). These earlier experiments were performed with peptides isolated from SDS-gels making the dem-

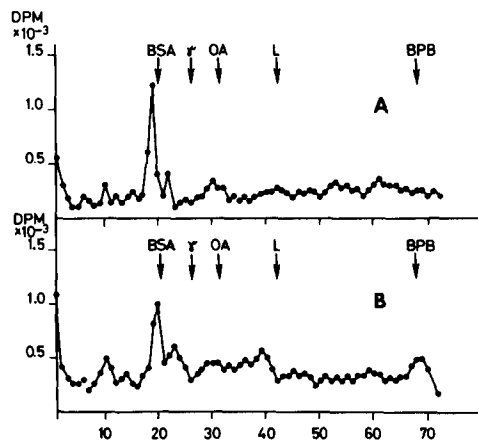


FIG. 2. SDS-gel analysis of rat and mouse T cell receptor material. The material was isolated from internally labeled supernates from Lewis anti-DA (A) and C57BL/6 anti-CBA MLC (B) via antiserum 801. After concentration, isolated material was analyzed under reducing conditions on 10% SDS gels and radioactivity in 1-mm slices was determined as described in Materials and Methods.

TABLE V  
Absorption of Rat and Mouse T Cell Receptor Material on Different  
Immunosorbents

Immunosorbent	Rat T cell re- ceptor material	Mouse T cell receptor materi- al
	dpm	dpm
Rabbit anti-rat Ig (polyvalent)	17,586	NT*
Rabbit anti-mouse Ig (polyvalent)	NT	17,974
Rabbit anti-RSA	17,721	NT
Rabbit anti-MSA	NT	17,438
DA anti-Lewis	16,835	NT
DA NS	16,429	NT
	NT	18,103
CBA anti-C57BL/6	NT	17,856
CBA NS	3,146	4,932

801

Internally labeled T cell receptor material was purified via 801 immunosorbent from Lewis anti-DA and C57BL/6 anti-CBA MLC supernate. 20,000 dpm in 0.3 ml were absorbed on a  $0.5 \times 3$ -cm immunosorbent for 3 h at room temperature and column was washed with 3 ml of PBS; the washing fluid was counted. RSA, rat serum albumin; MSA, mouse serum albumin. The indicated immunosorbents were prepared by coupling the antigens onto CNBr-activated Sepharose 4B following the instruction of the manufacturer (Pharmacia Fine Chemicals).

\* Not tested.

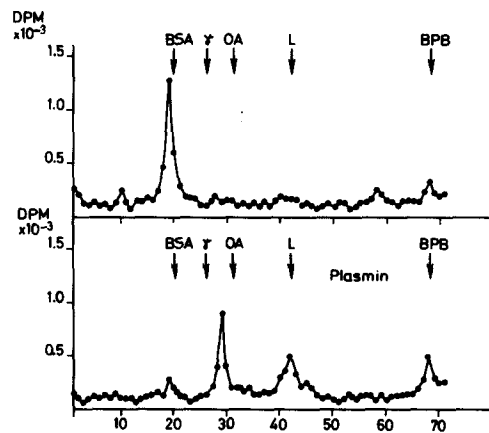


FIG. 3. Splitting of T cell receptor material with plasmin. The digestion procedure was performed as described in Materials and Methods using internally labeled Lewis anti-DA MLC supernate as a source of T cell receptor material. Analysis of the products was done on 10% SDS gels and radioactivity was determined in 1-mm slices as described.

onstration of antigen-binding capacity of the degraded material to cells difficult. We therefore exposed the present molecules that bound and eluted from 801 immunosorbent to plasmin, known to split Ig heavy chains within the constant region (37). Fig. 3 demonstrates such an experiment with material isolated from Lewis anti-DA MLC supernate and degraded as described previously. The material representing 70,000-

dalton peak molecules could be split into two main fragments of ~45,000 and 25,000 daltons (see also Fig. 5).

The next question we asked was whether we could localize the antigen-binding capacity into one of the two fragments. We therefore digested the  $^{125}\text{I}$ -labeled 70,000-dalton molecules isolated from Lewis anti-DA MLC supernates via 801 immunosorbent with plasmin and fractionated the products on G-200. The main peaks as determined by radioactivity were pooled, concentrated, dialyzed against PBS, and

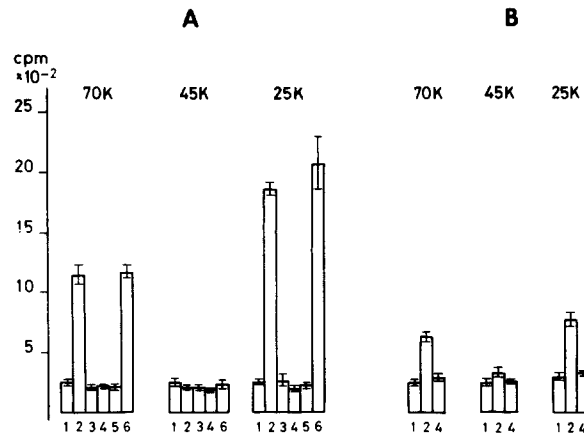


FIG. 4. Demonstration of antigen-binding capacity of T cell receptor material. Lewis anti-DA MLC supernate was purified over 801 immunosorbent, radiolabeled with  $^{125}\text{I}$  and digested with plasmin as described in Materials and Methods. Peptides were fractionated over G-200 Sephadex in 6 M urea and 0.1 M sodium acetate, pH 4.5. Fractions forming peaks of 45K and 25K as determined by counting the radioactivity were pooled, dialyzed, and concentrated, and 50,000 (A) or 100,000 (B) cpm were incubated with  $1 \times 10^6$  cells as indicated for 1 h at  $4^\circ\text{C}$ . Columns represent mean  $\pm$  SE of quadruplicates. 1 = Lewis, 2 = DA, 3 = August, 4 = BN, 5 = L.BN, 6 = AVN.

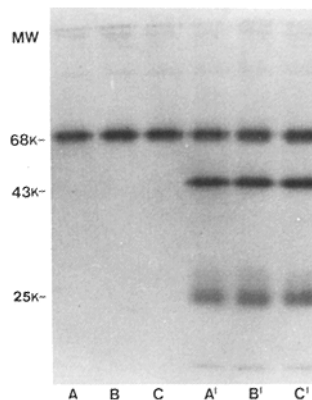


FIG. 5. Similar T cell receptor molecules on T cell subpopulations. C57BL/6 anti-CBA MLC T subpopulations were prepared as described in Materials and Methods and internally labeled supernate from these T cell subpopulations were absorbed on 801 immunosorbent. Isolated material was exposed to plasmin and the products were analyzed on 10% SDS slab gel. A, material from nonseparated cells; B, material from  $\text{Lyt-1}^-, 2^+, 3^+$  cells; C, material from  $\text{Lyt-1}^+, 2^-, 3^-$  cells. A', B', C', corresponding products after digestion with plasmin. Approximately 20,000 dpm were applied on A, B, C and about the double amount on A', B' and C'. Gel was exposed to Ilford film (Ilford Limited, Basildon, Essex, England) for 1 mo.

tested on rat spleen cells of different strains. Fig. 4 illustrates two successful experiments. The antigen-binding capacity could be localized into the 25K peptide.

*Similar or Identical T Cell Receptor Molecules Isolated from T Cell Subpopulations.* T cell subpopulations from C57BL/6 anti-CBA MLC were prepared as described previously. Part of these cells were used to check the purity of the T cell subpopulations. Using anti-Lyt sera in a protein A assay we found that the two subpopulations were not significantly contaminated with each other (data not shown). Internally labeled supernate (see Materials and Methods) from the subpopulations was absorbed on immunosorbent 801, and eluted material was analyzed on SDS-gels (see Fig. 5). The molecules isolated from a mixture of the population (nonseparated cells) (*A*), from Lyt-1<sup>-</sup>,2<sup>+</sup>,3<sup>+</sup> cells (*B*), and from Lyt-1<sup>+</sup>,2<sup>-</sup>,3<sup>-</sup> cells (*C*), were extremely similar if not identical in size when analyzed on a 10% SDS gel. All three types of molecules also gave similar split products when exposed to plasmin (*A'*), (*B'*), and (*C'*). These data strongly suggest that there must be very similar T cell receptor molecules on the two subpopulations recognized by antiserum 801.

*Functional Evidence That Antiserum 801 Reacts with T Cell Receptors.* We have previously demonstrated that anti-idiotypic antisera directed against T cell idiotypes can stimulate T cells into proliferation and even generation of cytolytic T cells with relevant specificity (38). The antiserum 801 is a heteroantiserum that reacts with the majority of normal T lymphocytes in mice and rats. Selective activation of antigen specificity would thus not be expected. Serum 801 was found to inhibit MLC reactions in concentrations >5% (data not shown). Using concentrations of ~0.5%, however, the antiserum was found to be a potent mitogen for normal T cells as demonstrated in Table VI. As seen, this mitogenic ability was in fact similar in strength to polyclonal T cell activators such as PHA or Con A and did occur with similar potencies on spleen cells as on purified T lymphocytes. No further analysis of the responding cells

TABLE VI  
*Induction of Proliferative Responses with Antiserum 801*

Cells	Serum or mitogen added	[ <sup>3</sup> H]TdR incorporation <i>Mean dpm ± SE of triplicates</i>
Aug S*	—	1,352 ± 103
Aug S*	801	102,272 ± 9,505
Aug S*	Rabbit anti-RSA	16,961 ± 984
Aug S*	Con A	271,182 ± 16,095
Aug S*	PHA	553,135 ± 16,402
Aug S*	LPS	141,425 ± 4,985
DA T‡	NRS	1,446 ± 122
DA T‡	801	483,798 ± 4,638
C57BL/6 T‡	NRS	1,772 ± 67
C57BL/6 T‡	801	201,381 ± 2,522

\*  $3 \times 10^5$  spleen cells in 200  $\mu$ l EHAA medium containing 0.5% fresh normal BN serum were incubated with 0.5% antiserum 801 or 0.5% rabbit anti-RSA in flat bottomed microtiter plates for 90 h and pulsed for 6 h with 1  $\mu$ Ci or [<sup>3</sup>H]TdR. Con A was used at a final concentration of 3  $\mu$ g/ml, PHA at 1  $\mu$ g/ml, and LPS at 10  $\mu$ g/ml.

‡  $5 \times 10^5$  DA or C57BL/6 T lymphocytes (prepared with Ig anti-Ig columns) were incubated under the same conditions in 200  $\mu$ l EHAA medium using either 0.5% fresh normal BN serum for the DA cells and 0.5% fresh C57BL/6 normal serum for C57BL/6 cells.

was carried out, except to establish via surface markers that they were of T cell type (data not included). We would thus conclude that serum 801 behaves in the anticipated manner with regard to triggering ability for T lymphocytes.

### Discussion

T lymphocytes are known to consist of several distinct subpopulations of immunocompetent cells that can be separated according to surface markers, pharmacologic activities, and antigen specificity requirements (39). With regard to the latter parameter, T lymphocytes are known to be obsessed with reactivity toward MHC antigens (40-42), and both helper and killer T cells frequently "see" antigen in the context of their own MHC structures in a manner quite distinct from B lymphocytes (43). The molecular details underlying the antigen-binding receptors of T cells are still confusing. Although by now most workers have been able to confirm our initial discovery that T and B lymphocytes reactive against the same antigens may share idiotypic determinants of presumed  $V_H$  type (9, 11, 12, 17, 21), analysis at the DNA level has so far failed to provide positive evidence for a  $V_H$ -like gene rearrangement in T cells similar to that occurring in B lymphocytes upon immunologic maturation (44). Analysis of antigen-binding receptors or antigen-specific factors isolated by various means have also yielded somewhat bewildering results. Our initial analysis of T cell receptors with specificity for MHC antigens in the rat indicated that a predominant structure (maybe the sole one) was a 70,000-dalton polypeptide chain (14). This chain could bind to antigen and express idiotypic determinants (14). During the last few years this finding has been confirmed by several workers using different systems including hapten-specific binding factors (45), sheep erythrocyte-specific suppressor molecules (23), and MHC-specific systems (17). In contrast, several other groups have reported on the common occurrence of antigen-specific factors consisting of two polypeptide chains, one of which could be coded for by the I-J region in the mouse (46), whereas the other shared features with the chains described in the present system.

Here we present results obtained with antisera raised in rabbits against idiotype-positive rat T receptor material with specificity for allogeneic MHC antigens. Such antisera have been produced by others using different experimental systems (47-51).<sup>2</sup> Specific antisera could be raised as judged by the selective reactivity with T lymphocytes and by the identical molecular features of molecules brought down by this xenoantiserum in comparison to earlier anti-idiotypic reagents (14). Some new findings were obtained. Previous studies using anti-idiotypic reagents had shown that similarly generated MLC T blasts of  $1^+, 2^-, 3^-$  and  $1^-, 2^+, 3^+$  phenotype carried distinctly different idiotypes, paralleling their distinctly different antigen-binding specificities (35, 52). With the present antiserum,  $Lyt-1^+, 2^-, 3^-$  and  $1^-, 2^+, 3^+$  blasts were both stained although the  $2^+, 3^+$  cells fluoresced more intensely. Due to the complexity of the reagent we cannot tell whether this represents a difference in quantity or quality of the receptors with regard to constant region epitopes. However, we failed to find any difference in size and susceptibility to plasmin degradation of the molecules

<sup>2</sup> Gershon, R. K., G. M. Iverson, C. A. Janeway, R. W. Rosenstein, R. E. Cone, H. Cantor, M. Fresno, J. A. Mattingly, H. Wigzell, H. Binz, H. Frischknecht, and W. Ptak. The isolation and characterization of T cell-derived molecules reacting with hetero-antisera against affinity purified antigen-specific T cell products. Manuscript submitted for publication.

being precipitated by the anti-T receptor antiserum from either blasts. The two groups of MLC-activated T blasts seem to carry antigen-binding receptors of very similar overall structure. We would then conclude that the xenoantiserum reacts with the constant region  $C_r$  of T cell receptors present on both  $\text{Lyt-1}^+, 2^-, 3^-$  and  $\text{Lyt-1}^-, 2^+, 3^+$  cells.

The present data also provide some new information on where the antigen-binding specificity resides in the degradation fragments of the 70,000 dalton molecules. Plasmin degradation yielded a 45,000- and a 25,000-dalton fragment in a manner similar to what is seen using heavy chain Ig molecules (37), and to what has been reported for an antiarsanilic specific polypeptide chain produced by a T hybridoma (J. Capra, unpublished data). Enough material was obtained for plasmin splitting to conclude that the 25,000-dalton fragment contain the antigen-binding specificity. This is again in agreement with two previous studies where antigen-specific factors from murine T cells split either by plasmin (J. Capra, unpublished data) or by "spontaneous" degradation (45), produced two fragments with the smaller one in the 20,000–25,000 dalton range expressing the antigen-binding capacity. We would thus conclude from the present studies with the xenogeneic anti-T receptor antiserum that the majority of rat and mouse T lymphocytes, regardless of  $\text{Lyt}$ -phenotype or antigen-binding specificity, express molecules with striking similarities of size and degradation properties. It is quite likely, however, that isotypic variability among the IgT molecules may exist in a manner analogous to what is known for B cell-derived immunoglobulin molecules.

The low frequency of positively stained T blasts obtained by PHA stimulation would be in favor of such a possibility. Likewise, the frequent occurrence of I-J-coded chains present in antigen-specific factors reported from T cells involved in suppressor circuits (46, 53) would argue in such a direction. Binding of MHC-coded chains to the 70,000-dalton chain may contain enough energy within self-MHC combinations using variable region sites to fully explain the presence of two such chain T-derived molecules without implying isotypic variability (54).

Besides having the expected cellular and molecular reactivity as measured by binding studies, the present antiserum also functioned as an expected T receptor reagent by performing as an extremely potent inducer of proliferation of normal mouse or rat T lymphocytes *in vitro*. This triggering of anti-T receptor antisera has been noted before (38, 55) in situations where anti-idiotypic reagents with unusual power initiated specific T cells to manifest immune functions of diverse natures. Availability of anti-T cell receptor sera of the present kind may allow a more refined analysis of the underlying requirements for this polyclonal triggering.

### Summary

Antisera specific for the constant part  $C_r$  of T cell receptor molecules in mice and rats have been produced in rabbits by immunization with idiotype-positive rat T cell molecules. Such antisera could be shown to react with the majority of normal T lymphocytes from both rats and mice. Mixed leukocyte culture-activated T blasts displayed a higher percentage of positive cells than concanavalin A- or phytohemagglutinin-induced T blasts. Lipopolysaccharide-induced B blasts were not reactive with the antiserum.  $\text{Lyt-1}^-, 2^+, 3^+$  blasts displayed a significantly brighter staining than the corresponding  $\text{Lyt-1}^+, 2^-, 3^-$  blasts. Isolation of the reactive molecules from T cells by

immunosorption yielded a 70,000-dalton single chain polypeptide as the dominant group of molecules. Plasmin caused the chain to split into 45,000- and 25,000-dalton polypeptides, with the smaller fragment displaying antigen-binding capacity. Molecules identical in size and plasmin degradation patterns were isolated from Lyt-1<sup>+</sup>,2<sup>-</sup>,3<sup>-</sup> and 1<sup>-</sup>,2<sup>+</sup>,3<sup>+</sup> blasts. Preliminary functional data supported the view that the antiserum is directed against the constant region C<sub>γ</sub> relevant receptor structures on immunocompetent T lymphocytes.

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