

SHARED IDIOTYPIC DETERMINANTS ON  
B AND T LYMPHOCYTES REACTIVE AGAINST THE SAME  
ANTIGENIC DETERMINANTS

III. Physical Fractionation of Specific Immunocompetent T  
Lymphocytes by Affinity Chromatography using Anti-Idiotypic  
Antibodies\*

BY HANS BINZ AND HANS WIGZELL

*(From the Department of Immunology, Uppsala University, Uppsala, Sweden)*

The eventual aim in the analysis of factors involved in the regulation of the immune response is to link structure with function. The molecules on B lymphocytes carrying antigen-binding specificity have been found to be more or less "conventional" immunoglobulins (1), the specificity of which are seemingly identical to that of their humoral products, the serum antibodies (2). A convincing procedure to prove this matter was via specific immunosorbent procedures, whereby selective depletion or enrichment of the relevant immunocompetent B lymphocytes could be achieved (3). In the analysis of the structures on T lymphocytes carrying specific antigen-binding capacity such approaches have been less productive. Using conventional immunosorbants, T lymphocytes seem to bind poorly for reasons not understood (3). So far the only regular way through which selective depletion of T lymphocytes according to immune reactivity has been obtained is via cellular immunosorbants, that is using antigenic cells as the immunosorbant material (4-6). By such assays selective depletion has been achieved but with poor enriching capacity (5).

Using anti-idiotypic antibodies we have previously been able to show that B and T lymphocytes reactive against the same antigens (in this case the major transplantation antigens of the species) express shared idiotypic determinants indicating certain shared peptide sequences in the antigen-binding areas of the respective antigen-binding receptors (7). The strength of the anti-idiotypic reactions with T cells was such that it allowed actual physical visualization of the respective idiotypic T and B lymphocytes (8,9). Such anti-idiotypic antibodies will selectively inhibit the binding of graft-vs.-host (GvH)<sup>1</sup>-reactive lymphocytes to the relevant allogeneic monolayer, indicating a direct linkage between idio-type and antigen-binding activity (10). To finally prove that the idiotypic T

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<sup>1</sup> Abbreviations used in this paper: Au, August; BME, Eagle's basal medium; FCS, fetal calf serum; GvH, graft-vs.-host.

lymphocytes as visualized by the anti-idiotypic antibodies are indeed the same cells that participate in specific T-cell-mediated immune reactions against the relevant antigen, specific affinity fractionation of idiotypic T lymphocytes have now been carried out. We used techniques involving anti-idiotypic antibody-coated T cells being selectively removed by anti-Ig columns, thereby allowing selective depletion or enrichment of the relevant idio-type-positive cells. By such approaches we have been able in the present article to obtain almost "pure" idiotypic T lymphocytes as judged by specific immune reactivity; that is, they react very well against the relevant antigen. When comparing the actual frequency of idiotypic cells in a population in relation to reactivity against the relevant antigen a striking positive correlation was found signifying a direct relationship between idiotypic and specific immune reactivity. The theoretical implications of the findings will be discussed.

### Materials and Methods

*Animals.* Rats of the inbred strains Lewis (L) (Ag-B<sup>1</sup>), DA (Ag-B<sup>4</sup>), August (Au) (Ag-B<sup>5</sup>), and BN (Ag-B<sup>3</sup>) as well as F<sub>1</sub> hybrids between some of these strains were domestically raised and maintained. Adult male or female rats were used within an experiment.

*Preparation of Rat Lymphoid Cell Suspensions.* Spleens and lymph nodes were teased with forceps in RPMI 1640 (Flow Laboratories, Inc., Rockville, Md.) complemented with 2.5 mg/ml of glutamine, 100 IU/ml of penicillin, 100  $\mu$ /ml of streptomycin, and 5% heat inactivated (30 min, 56°C) fetal calf serum (FCS) (Flow Laboratories, Inc.). The cell suspension was filtered through a net and the single cell suspension was washed once with the same medium. Red cells were lysed by incubation with 0.84% (wt/vol) NH<sub>4</sub>Cl for 8 min (including 6 min centrifugation at 800 *g*). Cells were washed again twice with medium. Cell viability was judged by trypan blue exclusion.

*Purification of Rat T Lymphocytes.* Rat T lymphocytes were purified by passing rat spleen and lymph node cells through Degalan beads previously coated with rat Ig and rabbit antirat Ig (11). In the present report 6  $\times$  10<sup>8</sup> spleen and lymph node cells at a concentration of 5  $\times$  10<sup>7</sup> cells/ml were passed through a 1.5  $\times$  30 cm Degalan column with a flow rate of 30 drops per min. Cells were harvested on ice and washed again with medium (see above).

#### *Preparation of Rat Anti-Idiotypic Antisera*

**SERUM POOL A.** 8 (Lewis  $\times$  BN)F<sub>1</sub> male rats were injected intraperitoneally on day 0 with 0.5 ml of phosphate-buffered saline containing 5  $\times$  10<sup>7</sup> male Lewis T lymphocytes (purified as described above). Animals received two more injections of 3  $\times$  10<sup>7</sup> and 5  $\times$  10<sup>7</sup> Lewis T cells, respectively, in 3-wk intervals and were bled by heart puncture 10 days after the last injection. The serum pool was heat inactivated (30 min, 56°C) and stored at -70°C.

**SERUM POOL 1003.** This pool was obtained from five (Lewis  $\times$  DA)F<sub>1</sub> rats after inoculation four to seven times with 2.5  $\times$  10<sup>7</sup> Lewis T lymphocytes in 3-wk intervals. Animals were bled by heart puncture 1 wk after each injection. This serum pool was handled as described for serum pool A. (For details on the anti-idiotypic tests of this serum pool see reference 7.)

**SERUM POOL 3.** This was obtained from four (Lewis  $\times$  DA)F<sub>1</sub> rats inoculated eight times with 2.5  $\times$  10<sup>7</sup> Lewis peripheral T lymphocytes in 3-wk intervals. The pool was obtained 6 days after the last immunization, heat inactivated at 56°C for 30 min, and kept at -70°C until use. Similar tests of anti-idiotypic specificity as for 1003 have been performed.

The three pools were absorbed extensively with F<sub>1</sub> lymphocytes to remove possible autoantibodies (12). All three serum pools showed specific binding to Lewis T lymphocytes in a radioimmunoassay described elsewhere (7). They also showed specific suppression of GvH reactions or mixed leukocyte culture responses as previously reported (7).

*Staining of Idiotypic Lewis T Cells against DA Antigens.* Cells were incubated at 0°C for 1 h with 1003 serum diluted 1:5 using RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, and 10 mM NaN<sub>3</sub>. The cells were then washed thrice, and incubated for a further 1 h with FITC-labeled rabbit-antirat Ig antibodies using a dilution at 1:10. The cells were subsequently read, after three washings, using a Leitz SM-Lux microscope equipped with a Ploem fluorescence-illuminator.

*Purification of Antigen-Reactive T Lymphocytes by the use of Anti-Idiotypic Antisera.*  $5 \times 10^8$  rat T lymphocytes (for preparation see above) were incubated with 2.5 ml of anti-idiotypic antiserum or F<sub>1</sub> normal serum for 1 h at 0°C with occasional shaking. Cells were washed five times with medium for 6 min at 800 g and adjusted to  $5 \times 10^7$  cells/ml. Cells were then passed through a 1 × 10 cm Degalan column, the Degalan beads previously coated with rat Ig, and rabbit-antirat Ig, using a flow rate of 35 drops/min. After the peak of the passed cells had come out an additional washing vol of 20 ml was allowed to pass through the column. The passed cells were harvested on ice, washed once with medium, and incubated with a second anti-idiotypic antiserum or normal F<sub>1</sub> serum as described above and passed afterwards again over a Degalan column under the same conditions as above.

The Degalan beads were removed from the column, placed into a beaker and stirred very gently with a Pasteur pipette after addition of some medium. The cells coming off the Degalan beads were washed twice with medium and kept at 0°C. In the same way cells were removed from the second Degalan column. All cells samples were finally adjusted to  $3 \times 10^7$  cells/ml Eagle's basal medium (BME) and kept at 0°C until used for the induction of GvH-reactions.

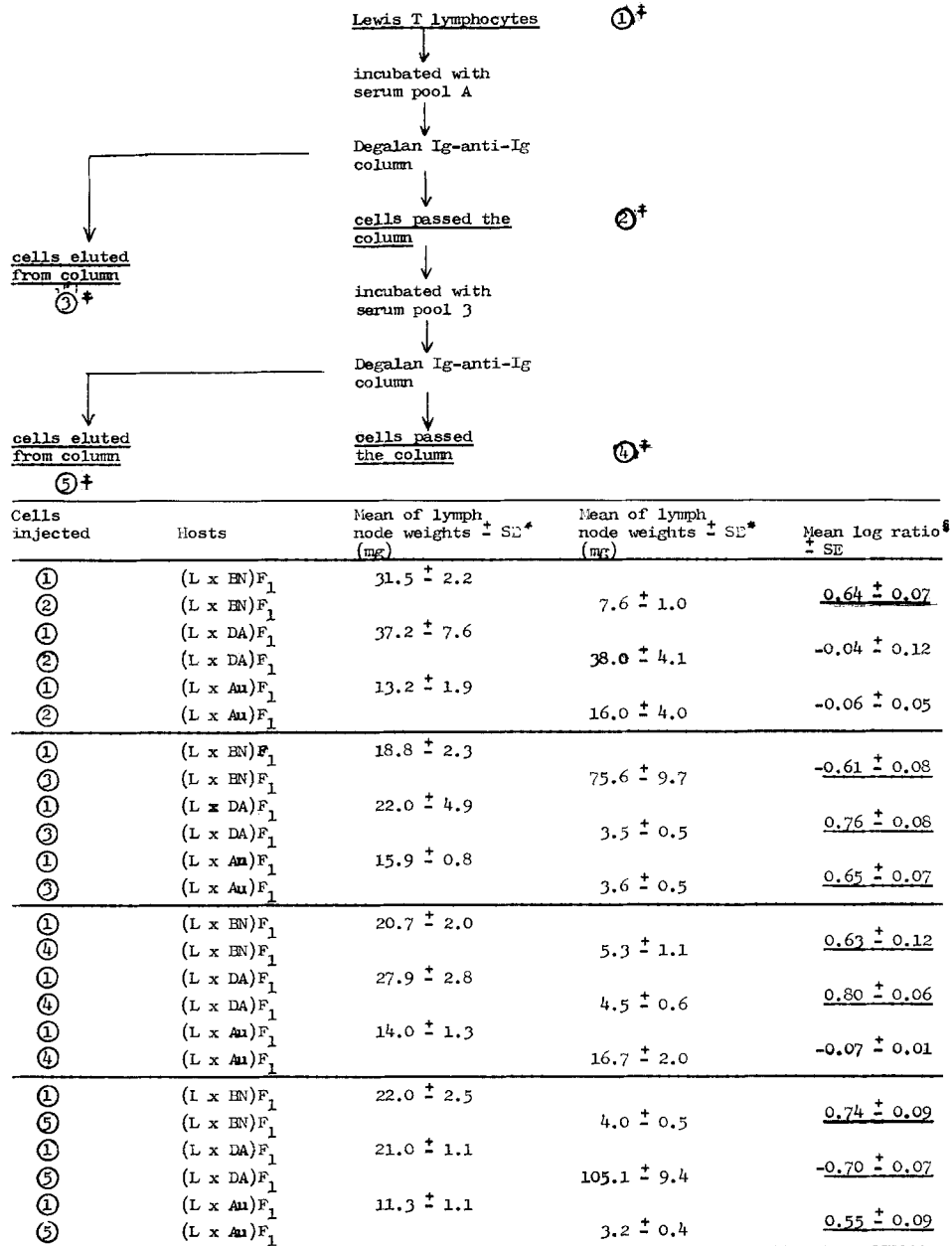
*GvH-Reactions.* These were induced in 5- to 10-wk old F<sub>1</sub> rats.  $3 \times 10^6$  (or as indicated in the tables) viable parental cells in 0.1 ml of BME were injected into each hind foot pad. Animals were killed 7 days later and the popliteal lymph nodes were excised and weighed (13).

## Results

*Selective Enrichment or Depletion of GvH-Reactive Normal T Lymphocytes Using Anti-Idiotypic Antibodies and Anti-Immunoglobulin Column Fractionation.* Mere incubation of T cells with anti-idiotypic antibodies reactive with the relevant antigen-binding, idiotypic receptors will, in our hands, have no impact on the future immune function of the T lymphocytes unless complement is added (7,11). Thus, we could use the following approach to selectively enrich for idiotypic T lymphocytes and to test for specific immune function of such cells using GvH assays. T lymphocytes from normal Lewis rats were purified from spleen and lymph node cell populations using anti-immunoglobulin column passage. Subsequently, such T lymphocytes were incubated with anti-idiotypic antisera reactive with idiotypic markers signifying reactivity with either DA or BN alloantigens (see Materials and Methods). The cells were then washed and filtered through a second anti-immunoglobulin column, making the assumption that idiotypic T lymphocytes due to their coating with immunoglobulin molecules (the anti-idiotypic antibodies) would now be selectively retained. The retained cells were then gently removed from the beads by mechanical means in a manner that has previously been found to allow recovery of functionally intact cells (14). The fact that the T cells had already once gone through an anti-immunoglobulin column would also allow for a more selective enrichment of the relevant T cells as most nonspecifically adhesive cells had already been removed (see recovery figures in Fig. 1). Passed and retained cells were then tested in the GvH assay against relevant as well as against party allogeneic combinations.

The present approach to obtain selectively depleted or enriched GvH-reactive cells could be shown to function in a satisfactory manner. In Fig. 1 is shown the results of such an experiment where two anti-idiotypic antisera were applied consecutively, both antisera achieving the expected selective retention of their respective potential GvH-reactive cells.

From the results in Fig. 1 we can deduce the following: Incubation of normal Lewis T lymphocytes with antisera specific for idiotypic markers indicating reactivity against BN alloantigens (serum A) followed by passage through anti-



\* Mean weights of three to ten lymph nodes.

† Cell numbers: 1=600x10<sup>6</sup>, 2=521x10<sup>6</sup>, 3=64x10<sup>6</sup>, 4=396x10<sup>6</sup>, 5=44x10<sup>6</sup>.

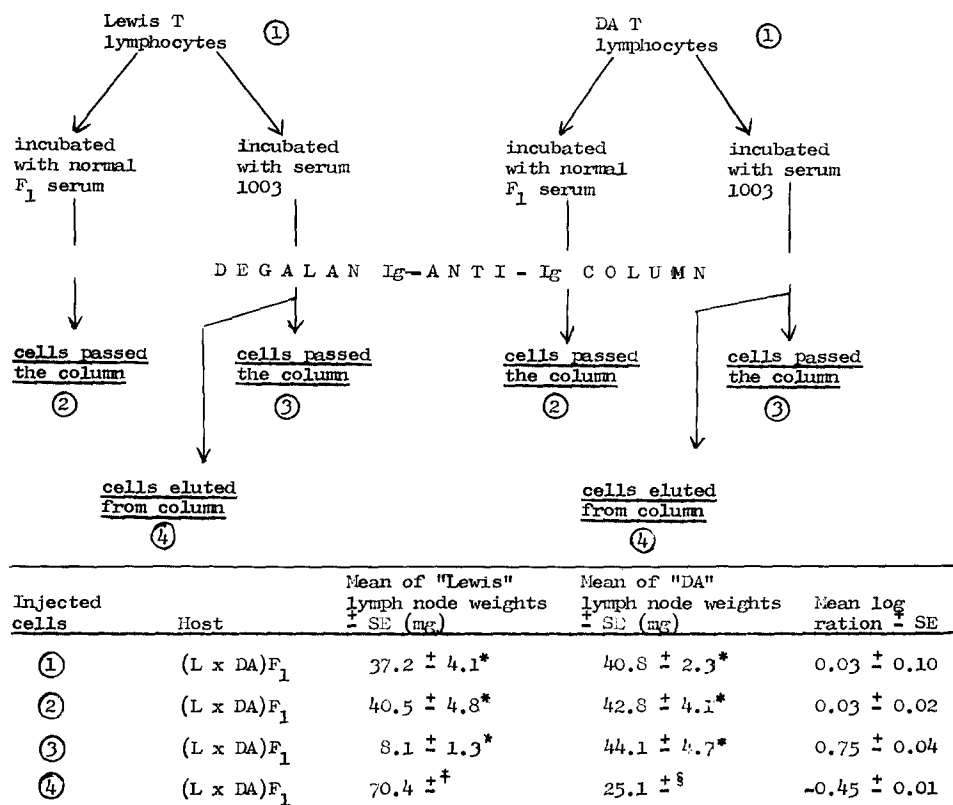
‡ Underlined values significantly different from 0.00

FIG. 1. Specific accumulation and depletion of two antigen-reactive T-cell populations by the use of two different anti-idiotypic antisera as measured in the local lymph node assay.

Ig column rendered the passed cells selectively unresponsive against BN alloantigens, whilst keeping the reactivity against DA and Au intact. The cells that were retained and mechanically eluted from the column beads could be shown to be highly significantly enriched for reactivity against BN, whereas no significant detectable reactivity was expressed against DA or Au. The control of nonpassed cells merely incubated with anti-idiotypic antibodies confirmed that mere incubation with anti-idiotypic antibodies will in no detectable manner perturb the future function of such idiotypic T lymphocytes (7). Some of the passed cells were then incubated with a second anti-idiotypic antiserum, this time with specificity for receptors against DA alloantigens (serum 3), followed by passage through yet another anti-Ig column. Now, the passed cells displayed no GvH reactivity for DA (and none for BN) but they displayed normal reactivity against Au antigens. The retained and mechanically eluted cells showed a highly increased specific reactivity against DA alloantigens. Yet another experiment with the same design and the same antisera was carried out yielding very similar results.

*Direct Positive Correlation between Percentage of Idiotypic Cells in a Given Cell Population and the Specific Potential Immune Reactivity.* We now had the means to both visualize the idiotypic T lymphocytes using fluorescent antibody techniques and anti-idiotypic antibodies (9) and to separate T lymphocytes according to function using anti-idiotypic antibodies in combination with anti-Ig column fractionation (see Fig. 1). Thus, experiments were carried out to actually calculate the percentage of idiotypic cells in a population in relation to specific immune reactivity. Using the design of anti-idiotypic antisera and anti-Ig columns as described before we thus allowed Lewis normal T lymphocytes to filter through anti-Ig columns after having been preincubated with either serum 1003 (reactive with anti-DA receptors) or normal rat serum. The unpassed, column-passed, or retained and eluted cells were then tested for percentage of cells with idiotypic markers of anti-DA specificity in relation to GvH reactivity against DA alloantigens. As shown in Fig. 2, it could be demonstrated that T cells incubated with anti-idiotypic antisera (like in Fig. 1) were selectively retained on the anti-Ig columns as indicated by the subsequent GvH assays. A small aliquot of the cells that were going to be used for GvH tests were taken aside for the fluorescent antibody tests. These revealed (see Table I) a highly significant depletion (close to background levels) of idiotypic cells subsequent to anti-idiotypic serum incubation and filtration through the anti-Ig column. A corresponding increase in the percentage of idiotypic T cells was found amongst the mechanically eluted cells from this anti-Ig column.

In the local popliteal assay strict mathematical formulas can be applied in the calculation of the relative immune potential of a given cell population (15). Using the slope calculations with regard to dose-response relationships that exist in the present system a relationship was formulated between the anti-DA reactivity of the control, passed or eluted cell populations obtained after anti-idiotypic incubation, and anti-Ig column fractionation and the percentage of idiotypic cells. A striking positive correlation was found between the percentage of "anti-DA" idiotypic-positive cells in a population and the GvH reactivity against DA (see Fig. 3).



Popliteal lymph node weight of normal F<sub>1</sub> animals between 5 and 10 mg.

\*3x10<sup>6</sup> viable lymphocytes were injected into each hind foot pad  
Mean weights of 3 to 10 nodes.

†1.1x10<sup>6</sup> viable lymphocytes were injected into each hind foot pad.  
Mean weights of 3 nodes.

‡1.5x10<sup>6</sup> viable lymphocytes were injected into each hind foot pad.  
Mean weights of 3 nodes.

FIG. 2. Purification of Lewis T lymphocytes reactive against DA alloantigens by the use of serum pool 1003 as measured in the local popliteal lymph node assay.

### Discussion

In the present article it is shown that normal T lymphocytes with potential immune reactivity against a given antigen can be physically separated from other cells by the use of anti-idiotypic antibodies and anti-Ig column fractionation. Selective depletion or enrichment of immunocompetent cells (as subsequently measured in quantitative GvH assays) against two distinct sets of antigens governed by the *Ag-B* locus could thus be achieved by applying in succession two different anti-idiotypic antisera. Using one such antiserum virtually all reactivity against one set of antigens could be removed without significantly affecting the reactivity of the remaining cells towards third-party antigens. This would thus indicate the existence of two largely nonoverlapping T lymphocytes with immune reactivity against the two sets of antigen. In further support of this were the results on the highly enriched cells (see Fig. 1)

TABLE I  
*The Percentage of Lewis T Cells Expressing "Anti-DA" Idiotypes as Measured by  
 Fluorescent Antibody Tests; Impact of Filtration Through Anti-Ig Columns After  
 Incubation with 1003 Serum*

Cells*	% Fluorescent cells‡
Lewis cells, incubated, not fractionated, 1003 serum	76/964 = 7.8 (6.3)
Lewis cells, incubated 1003, passed anti-Ig column	9/586 = 1.5 (0.0)
Lewis cells, incubated 1003, bound and eluted from anti-Ig column	154/469 = 32.8 (31.3)
Lewis cells, incubated F <sub>1</sub> normal serum, passed anti-Ig column	5/323 = 1.5

\* Lewis T lymphocytes already filtered once through one anti-Ig column before experiment. All cells developed using a FITC-rabbit-antirat Ig serum.

‡ Number of positive cells out of total counted. Figures within brackets indicated "true" percentage of cells with "anti-DA" idiotypic markers after subtraction of background B lymphocyte stained cells. Percentage of B cells after first and second anti-Ig column around 1.5%.

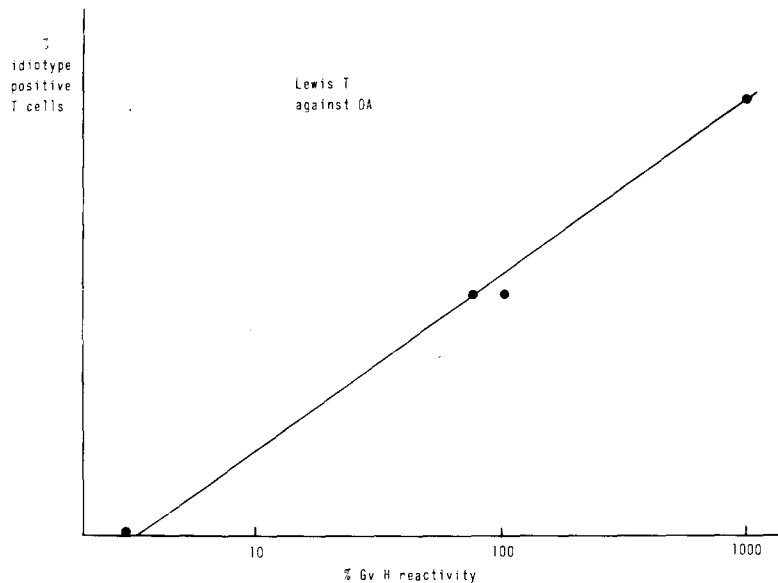


FIG. 3. Positive correlation between percent "anti-DA" idiotypic cells in populations of normal Lewis T lymphocytes and corresponding GvH activity against DA antigens. Cell populations as obtained from experiment depicted in Fig. 2. Calculations of linear regression yielded a figure for  $r^2$  of 0.99.

that were originally retained and then mechanically eluted from the anti-Ig columns. Such cells could be shown to act like virtually "pure" cells with regard to immune reactivity, displaying highly enriched reactivity against the relevant antigen(s) whilst showing no significant activity against third-party antigens. We would thus conclude that in the present antigenic system (Lewis-anti-DA, Lewis-anti-BN, and Lewis-anti-Au) any doubly reactive T lymphocytes must be very rare indeed.

We have previously been able to demonstrate that anti-idiotypic antibodies are quite able to selectively inhibit the idiotype-positive T cell from binding to its relevant antigen (10). As we now have the means to detect and enumerate idiotypic T (and B) lymphocytes via anti-idiotypic antibodies (9) we also wanted to see if it was possible to show any direct correlation between the number of idiotype-positive cells in a population and the immune capacity of the same cells against the relevant antigen. A highly significant positive correlation was found ( $r^2 = 0.99$ , Fig. 3) between the idiotype frequency in a cell population and the specific GvH reactivity of those cells. This we interpret to mean that in fact the idiotype-positive cells do comprise the actual specific GvH-reactive cells. In additional experiments we have achieved purity with regard to idiotypes in excess of 90% by the present approach, however, in the experiment depicted in Fig. 2 only 31% "purity" was obtained. The results in Fig. 1 with regard to exquisite specificity of the retained, mechanically eluted cells would argue for a higher degree of purity than 31% [now assuming that most, if not all, of the idiotype-positive cells can be visualized by our anti-idiotypic antisera (9)]. Whereas the cells passing through the anti-Ig column in the experiment depicted in Fig. 2 had sizeable nonspecific trapping of cells thus allowing dilution of the specifically retained cells (around 20% of the applied cells were retained), less nonspecific retention was observed in the experiment in Fig. 1. Here, the degree of retention was 10%, closer to an expected figure would mostly specific, idiotype-positive cell be retained (9). Furthermore, we believe that cells with adhesive properties in general would be discriminated against in the mechanical elution procedure, thus favoring the enrichment of "anti-idiotypic-retained" cells in such a population.

Irrespective of whether all T lymphocytes with potential reactivity against a given antigen can be visualized or not using anti-idiotypic antibodies (for discussion of idiotype on T cells see references 7 and 9) the present approach allows the physical isolation of highly enriched, functionally "pure" T lymphocytes. Biochemical and functional studies using such immunocompetent T-cell populations would now seem a fruitful field of exploration.

### Summary

Anti-idiotypic antibodies made against antigen-binding receptors on T lymphocytes with specificity for certain *Ag-B* locus antigens selectively react with T lymphocytes with potential immune reactivity against the very same *Ag-B* antigens. This was shown by affinity chromatography of normal Lewis T lymphocytes on anti-Ig columns after contact with the relevant anti-idiotypic antiserum. Here, it could be shown that incubation of the cells with an anti-(Lewis-anti-BN) antiserum caused subsequent selective retention of potential graft-vs.-host (GvH)-reactive cells against BN on the anti-Ig column, whereas Lewis T cells with reactivity against DA or August (Au) (carrying distinct *Ag-B* antigens in comparison to BN) passed through. The retained cells could be eluted and shown to display highly increased reactivity against BN with virtually no reactivity left against DA or Au antigens. Analogous results were obtained using an anti-(Lewis-anti-DA) antiserum.

The anti-idiotypic antibodies can be used in fluorescent antibody tests to



directly visualize the idiotype-positive cells. Using the separation design described above we analyzed selectively enriched or deleted T lymphocytes for presence of idiotypic cells as well as specific GvH reactivity. A highly significant positive correlation was found between percentage of a given idiotype in a population of T cells and the relevant GvH potential of the same population. From this, we deduce that the idiotype-positive T cells that can be visualized are indeed the very same T cells that express immune reactivity against the expected antigens.

The present data would thus directly demonstrate the existence of a largely nonoverlapping population of immunocompetent T cells capable of reacting against the various *Ag-B* locus antigens in the rat. Highly purified, functionally intact immunocompetent T lymphocytes with restricted immune reactivity can thus be produced from normal lymphocyte populations for further analysis.

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