Isolation of a T-cell clone that reacts with both antigen and anti-idiotypic: Evidence for anti-idiotypic as internal image for antigen at the T-cell level

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Communicated by Niels K. Jerne, February 10, 1987 (received for review November 5, 1986)

ABSTRACT T-cell lines were derived from ferredoxin nonresponder B10.D2 mice that share an idiotype expressed by a monoclonal antibody (Fd-B2) with specificity for one of the two major antigenic determinants (the C determinant) of the antigen. The T-cell line and T-cell clones derived from it release interleukin 2 not only in the presence of anti-Fd-B2 idiotype antibody but in the presence of ferredoxin. The line was shown to be major histocompatibility complex-restricted in that it would respond to the anti-idiotypic and antigen only in the context of presentation by cells of the *H-2^d* haplotype. This observation also establishes that the nonresponder status of *H-2^d* animals cannot be attributed to a lesion at the level of antigen presentation. Analysis of the fine specificity of one idiotypic clone showed that it responded only to the anti-idiotypic or products of the antigen containing the C determinant, since enzymatically degraded peptides devoid of this determinant did not stimulate these cells. Furthermore, it was found that presentation of both the antigen and the anti-idiotypic to the specific clone could be blocked by the Fd-B2 monoclonal antibody.

The concept that anti-idiotypic (Id^-) antibodies might structurally resemble the antigen from which the corresponding idiotypic (Id^+) antibody was derived is not new and has indeed been predicted (1, 2). Somewhat surprisingly, proof of this prediction has not been easy to establish and there are only a few reported cases in which Id^- antibodies have been shown to possess this property. Support for this concept was provided when it was found that Id^- antibodies to a variety of hormone-specific antigens were able to mimic hormone-specific effects, suggesting that internal images may exist within the immune system that reflect molecular structures outside the immune system (3, 4). Further examples of anti-idiotypic antibodies as internal images of antigen have been provided in a few other instances (5, 6). It is well-documented that T cells bear idiotypic determinants recognizable by anti-idiotypic antibodies directed to idiotypes expressed on immunoglobulins. The now classic experiments of Eichmann and colleagues with the A-CHO system (A5A-id) showed that antigen (A-CHO)-primed T and B cells could produce A5A Id^+ plaque-forming cells *in vitro* after stimulation with anti-idiotypic antibodies (7). Evidence that T cells could recognize anti-idiotypic as the internal image of antigen was demonstrated by Ertl *et al.* (8), who showed that cytolytic T lymphocytes specific for reovirus type-3-infected cells could lyse a B-cell hybridoma secreting an anti-idiotypic antibody specific for a monoclonal antibody directed to the reovirus hemagglutinin.

The ferredoxin (Fd) molecule is a small defined polypeptide that immunologically has only two antigenic determinants as assigned by specific antibody, one located within the

NH_2 -terminal heptapeptide (N determinant), and the other within the COOH-terminal pentapeptide (C determinant) (9). Nonresponder status in mice is associated with the *H-2^d* haplotype and maps to the I-A region (10). Monoclonal antibodies to both determinants have been generated in this laboratory. One such monoclonal, Fd-B2, which reacts with the C determinant has been studied in depth, since it was found that it was capable of reacting with, and eliminating, in the presence of rabbit complement, a population of T-suppressor cells in high-responder (B10.BR) mice (11). Further experiments with Fd-B2 and an anti-idiotypic antibody raised to it in rabbits demonstrated the presence of populations of Id^+ and Id^- T-suppressor cells with high connectivity in nonresponder *H-2^d* mice. The removal of either population resulted in the conversion of nonresponder mice to the responder status. Connectivity between these populations was proved by showing that mixing Id^+ - or Id^- -depleted populations in adoptive-transfer experiments effectively restored the nonresponder status to *H-2^d* animals (12).

The present study was undertaken to determine whether T-cell lines and clones expressing the Fd-B2 idiotype could be produced using nonresponder B10.D2 T cells, whether the Id^+ cells thus produced could respond specifically to both Fd and anti-idiotypic antibodies, and whether such reactivity could be blocked by the Fd-B2 idiotype. Our results show definitively that the Id^- antibody does indeed mimic the antigen Fd in the context of antigen recognition by T cells and that presentation of both antigen and Id^- antibody to reactive clones can be blocked specifically by the appropriate idiotype.

MATERIALS AND METHODS

Experimental Animals. B10.D2, B10.BR, C57BL/6, DBA/2 mice (used at the age of 5–12 weeks) were either purchased from The Jackson Laboratory or were bred from mating pairs (obtained from The Jackson Laboratory) and maintained in our animal facility. Anti-idiotypic antibody and normal rabbit immunoglobulin were obtained from New Zealand White female rabbits obtained from the University of British Columbia Animal Care Unit.

Antigens. Fd was purchased from Sigma (F7629), and partially purified protein derivative from *Mycobacterium tuberculosis*, used as a control antigen since animals were primed with Fd in complete Freund's adjuvant, was obtained from Connaught Laboratories (Willowdale, ON).

Anti-idiotypic Antibody. Anti-idiotypic antibody was raised by repeated immunization of rabbits with the Fd-specific monoclonal antibody as described (11). Briefly, specific anti-idiotypic antibody was purified by sequential affinity purification over Fd-B2 immunoadsorbent columns followed

by absorption on immunoadsorbent columns containing normal mouse immunoglobulin.

Monoclonal Antibodies. The Fd-B2 monoclonal antibody has been described (11). Briefly, it is produced by a hybridoma between B10.BR Fd immune splenocytes and SP/2 cells. It was grown up as ascites in pristane-treated irradiated mice and purified over DEAE Sephacel. Control monoclonal antibody of the same isotype was treated in an identical manner.

Immunization. Sublethally irradiated (500 rads; 1 rad = 0.01 Gy) Fd nonresponder B10.D2 mice were reconstituted by intravenous administration of 4×10^7 syngeneic, T-cell enriched splenocytes passed through nylon wool that had been treated with the Fd.B2 idiotype plus rabbit complement (to eliminate anti-idiotypic T cells) as described (12). Animals were also reconstituted with 4×10^7 B-cell (anti-thy-1 plus C)-enriched syngeneic splenocytes and rested for 7 days. They were subsequently primed by hind footpad immunization with 25 μ g of Fd in complete Freund's adjuvant (per footpad). Ten days later, popliteal and inguinal lymph nodes were removed for the establishment of T-cell lines and clones.

T-Cell Culture and Cloning. Lymphocytes from anti-idiotypic-depleted Fd-primed reconstituted B10.D2 mice were cultured at a concentration of 10^5 cells per ml in a total volume of 15.0 ml in Falcon tissue culture flasks (13). Culture conditions involved 50 μ g of Fd per ml, 2.5×10^6 irradiated (3600 rads) B10.D2 splenocytes per ml in RPMI 1640 medium with 10 mM Hepes/50 μ M 2-mercaptoethanol/10% heat-inactivated normal human serum (obtained from the Canadian Red Cross Blood Bank, Vancouver, BC). Cultures were incubated at 37°C in a 10% CO₂/90% air humidified incubator for 9 days and harvested. Live cells were separated by centrifugation on Lympholyte-M (Cedar Lane) and washed twice in RPMI 1640 medium with 3% fetal calf serum. Viable cells from the interphase were recultured for a further 9 days in RPMI 1640 medium containing 10 mM Hepes, 2 mM 2-mercaptoethanol, streptomycin (100 μ g/ml), penicillin (100 units/ml), and 10% normal human serum plus 100 μ g of Fd per ml and the feeder layer as described above. This cycling was repeated three times. Cells were cycled one more time in medium as described above except that anti-idiotypic antibody instead of Fd was added.

Cells growing under the above conditions were cloned by limiting dilution according to a modification (9) of the procedure described by Kimoto and Fathman (14). Briefly, cells were cloned into 200- μ l wells containing RPMI 1640 medium with 10% normal human serum and anti-idiotypic (50 μ g/ml) following a 4-day pulse of 1% interleukin 2 (IL-2)-containing medium [produced as described (15) from EL-4 phorbol myristate acetate-stimulated cultures]. Wells in which unmistakable single clones were visible were scaled up to 10-ml cultures containing anti-idiotypic antibody after the growing clone had covered between 1/2 and 2/3 of the bottom of the well.

IL-2 Release Assay. T cells from primary and established cell lines and clones were tested for IL-2 release in the presence or absence of the test antigens. Assays were carried out in duplicate. Each culture contained either 10^4 or 10^5 cells (usually 10^5 cells, except in conditions such as primary cloning in which limiting cell numbers did not permit the use of the higher number) and 10^6 irradiated splenocytes in a volume of 200 μ l. The medium (RPMI 1640) contained 35 μ g of antigen per ml, 10% normal human serum, and 50 μ M 2-mercaptoethanol. Cultures were maintained for 48 hr, and then supernatants were harvested and centrifuged at $500 \times g$ twice, with changes of centrifuge tubes each time to minimize the chances of contamination with cultured cells. Supernatants were tested on Con A-stimulated blasts prepared as described (16). Blast cells were distributed in 96-well micro-

titer tissue culture plates (Flow Laboratories) at 2×10^5 cells per ml in 0.1-ml aliquots in RPMI 1640 medium containing 2.5% fetal calf serum. To each well, 0.1 ml of the test culture supernatant was added. Doubling dilutions of the test material in the medium were used in each assay ranging from 1:2 (final dilution, 1:4) to 1:16. After 48 hr [³H]thymidine (New England Nuclear) was added at a concentration of 1 μ Ci per 10 μ l (1 Ci = 37 GBq) per culture, and cells were harvested 18 hr later on a MASH cell harvester. Thymidine incorporation was measured in a TRI-CARB 4550 Packard scintillation counter. With each experiment, a standard curve was established using the test cells and dilutions of a standard EL-4 IL-2-containing culture supernatant. Since essentially all assays gave similar results in terms of stimulation or background, results are presented as cpm rather than in terms of units established from standards. Positive reactions were equivalent to dilutions of EL-4 supernatants between 1:100 and 1:1000 and negative tests (3000–5000 cpm) were equivalent to EL-4 dilutions between 1:10,000 and 1:100,000.

RESULTS

Initial attempts were made to select idiotype-bearing (Id⁺) T cells by using anti-idiotypic antibody. Mice immunized with Fd were sacrificed, and T-cell-enriched populations were cultured for 9 days in the presence of 50 μ g of Fd per ml, harvested and tested for IL-2 release in the presence of anti-idiotypic antibody, Fd, or other control materials. The results of the assay (Fig. 1) show that T cells proliferating under these conditions produced significant levels of IL-2 in the presence of both Fd and the anti-idiotypic but not in the presence of IgG purified from nonimmune rabbits, irrelevant antigen, or no antigen.

The cell line established in this way was further tested for its ability to respond to the anti-idiotypic and Fd when antigen-presenting cells were allogeneic or of the same haplotype as the line. The results (Fig. 2) show clearly that the line responds only to anti-idiotypic or Fd as opposed to IgG from nonimmune rabbits, and irrelevant antigen only when the anti-idiotypic or antigen is presented in the context of the syngeneic H-2^d haplotype.

The cell line was cloned by limiting dilutions as described and individual clones were tested for their ability to respond to anti-idiotypic. It can be seen, from the examples shown in Fig. 3, that some clones responded well, whereas others did not. The C2 clone was selected for further study. Because the main aim of this study was to determine whether Id⁺ T cells could respond to the internal image (antigen-like) of the anti-idiotypic as well as to the antigen, experiments were done to establish whether C2 clone could respond specifically to both stimuli. These experiments (Fig. 4) showed that the clone responded equally well to Fd as it did to anti-idiotypic, and not significantly to IgG from nonimmune rabbits or irrelevant antigen, thus establishing the similarity of the topography between the antigen (Fd) and the anti-idiotypic recognized by T cells.

The anti-idiotypic used in these experiments was raised against a Fd-specific monoclonal antibody, Fd.B2, which reacts exclusively with the antigenic determinant located within the COOH-terminal pentapeptide of the Fd molecule (C determinant). Because we have shown in earlier studies that specific enzyme cleavage of the Fd molecule can selectively eliminate either the C or N determinant to produce functionally monovalent peptides, the C, N, or M fragments (M being doubly digested and devoid of both the N and C determinants), these reagents were used in the IL-2 release assay with clone C2 to further establish its fine specificity. The results (Fig. 5) verify previous observations presented here in that the clone responds exclusively to

FIG. 1. IL-2 release in culture supernatants of a Fd-dependent T-cell line. T cells (10^5) were incubated in complete RPMI 1640 medium supplemented with 10% normal human serum for 48 hr with 50 μ g of anti-idiotypic antibodies per ml to the idiotype Fd-B2 (anti-idiotypic; $\circ$ --- $\circ$); Fd ($\square$); normal rabbit immunoglobulin from nonimmune rabbits ($\blacktriangle$); partially purified protein derivative from *M. tuberculosis* ($\bullet$); or no antigen ($\circ$ — $\circ$). Source of antigen-presenting cells was irradiated (3600 rads) splenocytes from B10.D2 mice at 10^6 cells per ml. All assays were performed in duplicate in 200- μ l cultures. Harvested culture supernatants were tested in duplicate for the presence of IL-2 activity using 48-hr Con A-induced splenocyte blasts (2×10^5 cells per ml in 200 μ l) in the presence of [3 H]thymidine (1 μ Ci per 10 μ l per culture) by doubling dilution titration starting at 1:2 (final dilution, 1:4) to 1:16. Con A blasts were harvested 18 hr later using a MASH cell harvester and cell proliferation was measured as cpm. SD for data points did not exceed 10%.

either the intact molecule or the C fragment, and not at all to either the N or M fragments.

Finally, experiments were carried out to examine the ability of the idiotype (Fd-B2) to interfere with the ability of syngeneic antigen-presenting cells to present a combination of anti-idiotypic and Fd to the C2 clone, since it was argued that if the clone was recognizing an equivalent topography of these molecules, then the idiotype should also recognize the same epitope presented. The results (Fig. 6) are unequivocal in that they show essentially complete blocking of IL-2 release when antigen-presenting cells pulsed with both anti-idiotypic and Fd are in the presence of the Fd-B2 monoclonal antibody. Control cultures containing an irrelevant monoclonal antibody of the same isotype as Fd-B2 are not inhibited at all. Earlier studies in which anti-idiotypic (Fd-B2 reactive) T-cell lines were raised have shown that Fd-B2 does not possess nonspecific inhibitory properties to T-cell growth and IL-2 release (data not shown).

DISCUSSION

We have used a somewhat unusual protocol to demonstrate that idiotypes on T cells express epitopes that are topographically similar to immunoglobulin idiotypes and will respond specifically to both the antigen and anti-idiotypic antibodies.

FIG. 2. Major histocompatibility complex restriction of the Fd-dependent T-cell lines. Antigen-presenting cells were derived from B10.BR ($H-2^b$) (a), C57BL/6 ($H-2^b$) (b), DBA/2 ($H-2^d$) (c), and B10.D2 ($H-2^d$)-irradiated splenocytes. Assay conditions were the same as in Fig. 1. Cells were incubated with the antigens listed above and culture supernatants (final dilution, 1:4) were tested for IL-2, as shown as cpm [3 H]thymidine uptake by Con A blasts. SD for data for each bar did not exceed 10%. Bars: 1, no antigen; 2, partially purified protein derivative from *M. tuberculosis*; 3, IgG from nonimmune rabbits; 4, Fd; 5, anti-idiotypic.

T cells derived from adoptively transferred Fd-primed nonresponder mice that had been converted to responsiveness by selective removal of an anti-idiotypic T-suppressor cell population as described earlier (12) were cultured first in the presence of the immunizing antigen, Fd, and then tested for reactivity with anti-idiotypic antibody in a standard IL-2 assay. It was clear that a significant population of cells in the line were expressing the Fd-B2 idiotype since IL-2 release in the presence of either anti-idiotypic or Fd was equivalent (Fig.

FIG. 3. Cloned T-cell lines were tested for IL-2 production at a concentration of 5×10^4 cells per ml in the presence of Fd (bars 1 and 2), anti-idiotypic (bars 3, 5, 7, and 9), or IgG from nonimmune rabbits (bars 4, 6, 8, and 10). Representative results from various clones are shown. Assay conditions were as in Fig. 1. Antigen concentrations were at 35 μ g/ml. SD did not exceed 10%.

FIG. 4. IL-2 release by culture supernatants of clone C2 in the presence of anti-idiotypic (○---○), Fd (□), IgG from nonimmune rabbits (▲), partially purified protein derivative from *M. tuberculosis* (●), no antigen (○—○). Antigen concentrations were at 35 μ g/ml. SD for individual data points did not exceed 10%.

1). Experiments with both Fd and anti-idiotypic further showed that presentation of either material was stringently restricted, since the line would respond only when irradiated splenocytes from *H-2^d* mice (as opposed to *H-2^k* and *H-2^b*) were present. These results establish that the nonresponder

FIG. 5. Fine specificity of clone C2. Cells were grown in the presence of the reagents (35 μ g/ml), and culture supernatants were tested at a final dilution of 1:4 for the presence of IL-2. SD did not exceed 10%. Bars: 1, no antigen; 2, partially purified protein derivative from *M. tuberculosis*; 3, N determinant; 4, M determinant; 5, C determinant; 6, Fd.

FIG. 6. Ability of the Fd-B2 idiotype to block antigen presentation and subsequent IL-2 release by the C2 clone. Cells were incubated in the presence of anti-idiotypic and Fd (17.5 μ g/ml each for a total of 35 μ g of antigen per ml) or IgG from nonimmune rabbits (NRIg) and partially purified protein derivative from *M. tuberculosis* (PPD) and the Fd-B2 idiotype or a control monoclonal antibody CAMAL-1 (100 μ g/ml). SD did not exceed 10%. Bars: 1, no antigen; 2, anti-idiotypic + Fd; 3, anti-idiotypic + Fd + Fd-B2; 4, anti-idiotypic + Fd + CAMAL-1; 5, PPD + NRIg + Fd-B2; 6, PPD + NRIg + CAMAL-1.

status of *H-2^d* mice to Fd is independent of the ability of antigen-presenting cells to present antigen in the appropriate context.

Our studies with a clone (C2) derived from the T-cell line has shown clearly that Id⁺ T cells are capable of responding equally well to both anti-idiotypic antibody and antigen and that antigen recognition is dependent on the presence of the appropriate antigenic determinant (the C determinant). These findings establish definitively that the idiotype expressed, presumably by the T-cell receptor molecule, is topographically analogous to idiotypes expressed on antibody molecules (the Fd-B2 idiotype in this case).

Previously, we have shown that although the Fd-B2 idiotype is rarely expressed as a B-cell product, it represents a major idiotype expressed on regulatory T cells (11, 12). At a superficial level, it might appear that these observations are at variance with the findings of Coutinho and co-workers (17), who showed that major idiotypes in the B-cell repertoire (directed to trinitrophenyl-self) contribute to the selection of the T-helper cell repertoire. They established this by showing that anti-idiotypic antibody could block helper T-cell lines derived from conventional BALB/c mice but could not block analogous T-helper cell lines derived from anti- μ -treated animals that did not express the major TNP idiotype at the B-cell level. In our results, we have shown that the Fd-B2 idiotype on T cells is expressed in the absence of a major population of Fd-B2-expressing B cells. However, the Fd-B2-expressing T-cell lines and clones were derived from adoptively transferred animals whose T cells had been selectively depleted of anti-Fd-B2 idiotype-bearing populations by *in vitro* treatment with idiotypic antibody and complement (ref. 12 and this work). This procedure presumably results in the uncoupling of the regulatory idiotypic network and permits a response to Fd. This manipulation has a selective effect on idiotype expression in that the elimination of anti-idiotypic cells will favor the proliferation of the Id⁺ population. The results described by Coutinho and co-workers show that in their antigen system, idiotype expression on B cells enhances the expansion of Id⁺ T-helper

cells, through an as yet unexplained mechanism (thymic or peripheral) but presumably involving anti-idiotypic cells also. In our system, the Fd-B2 idotype is not expressed at the B-cell level, possibly as a result of suppression by an anti-idiotypic population. In the absence of precise information regarding the mechanism of idiotypic selection, it would be inappropriate to conclude that our findings are incompatible with those of Coutinho and co-workers, since we show that idiotypic repertoire on T cells can be influenced by network interactions (idiotypic or anti-idiotypic). There are, no doubt, a number of factors that selectively influence the expansion of individual T-cell clones, idiotypic-bearing B cells being one such interaction. Our results are consistent with the proposal that the T-cell idiotypic repertoire may mimic the B-cell repertoire in the context of the Fd-B2 idiotypic (17). However, it is worth mentioning at this point that there is considerable evidence showing that T cells frequently recognize antigenic epitopes that do not represent major epitopes recognized by B cells, and that with many antigenic systems, no predominant idiotypes at the B-cell level are expressed. These observations suggest that minor B-cell idiotypes could also have an influence on the selection of T-cell idiotypes: also, given the flexibility of the immune system, it is not unreasonable to suggest that in some antigenic systems T-cell network interactions may take place that are independent of B-cell networks and that these also may influence repertoire selection.

The observations that the Fd-B2 idiotypic was expressed by the C2 T-cell clone led us to predict that this idiotypic was capable of recognizing antigen (or anti-idiotypic) as it is presented on the surface of antigen-presenting cells. It has been difficult in the past for investigators to demonstrate that anti-antigen antibodies can block the presentation of antigen to sensitized T cells. The reason for this may be that B cells preferentially recognize epitopes not presented at a high frequency by T cells. Recently, some success with blocking antigen presentation with anti-antigen antibodies has been achieved with both murine and human T-cell clones using the defined antigens cytochrome *c* and a synthetic peptide derived from the influenza virus hemagglutinin (18, 19). Our findings corroborate these earlier observations in that the Fd-B2 monoclonal antibody can effectively block and eliminate the presentation of Fd to the C2 clone. In terms of network, our blocking studies take these observations further in that Fd-B2 was also found to be capable of blocking presentation of the anti-idiotypic to the C2 clone.

The results reported here show definitively that anti-idiotypic antibodies can stimulate antigen-specific clones equally as well as the immunizing antigen. Thus, the concept that anti-idiotypes represent structures analogous to antigen is supported at the level of the T-cell receptor.

This work was supported by Grant 68-3411 from the Medical Research Council of Canada.

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