Clonal Analysis of the Human B-Cell Repertoire Using a Heteromobility Assay

DEEPAK TEWARI*

Aaron Diamond AIDS Center, New York, New York 10016

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A heteromobility duplex tracking assay was developed to analyze B-cell clonality. The assay was based on the genetic variability of B-cell immunoglobulin (Ig) sequences. Binding of amplified (Ig) sequences to a single-stranded radiolabeled Ig DNA probe resulted in the formation of heteroduplexes. The mobilities of these heteroduplexes helped to distinguish clonal B cells.

The human immune system is characterized by expansion of the B-cell repertoire following recombination events that take place at both the genetic and the somatic levels. This expansion leads to the generation of an extremely diverse set of B cells. Diversity among B cells makes it difficult to devise suitable strategies for study of the B-cell clonal response (1). Like B cells, T cells are also very diverse. Suitable assays for analysis of clonal T cells have only recently become available (8, 11, 12). T-cell assays are based on the known heterogeneity of the T-cell receptor variable (V) regions and use techniques like single-stranded conformation polymorphism analysis (8) and the heteroduplex tracking assay (HTA) (11). T-cell studies have recently become more selective and narrow because of the associations that have been seen between specific T-cell gene subfamilies in health and disease (8, 11, 12). Such information about B-cell gene subfamilies is not available, and no attempt has so far been made to develop assays that can be used to study B-cell clonality. Most of the studies that have been done to analyze human clonal B cells have used strategies that involve sequencing of the variable regions of heavy-chain (VH) genes and light-chain genes and comparisons of the lengths of the complementarity-defining regions (2, 4, 6, 9, 10).

In this study, I have applied the principle of HTA to analyze B-cell clonality. The basis of HTA was the heteromobility of DNA duplexes on the gels. The heteroduplexes were formed due to annealing of diverse immunoglobulin (Ig) genetic sequences to a single-stranded (ss) gene family-specific probe. HTA has previously been shown to be a better technique for the study of genetic heterogeneity, mainly because of its ease of operation and unbiased approach. It does not involve sequencing of every Ig clone, which can be labor-intensive and time-consuming. One other advantage of HTA is that the observed bands on the gel can be directly correlated to the number of different clones in a given population (5, 11).

The assay was performed with peripheral blood mononuclear cells (PBMCs; 5 \times 10^6). PBMCs were obtained from leukocyte-enriched blood collected at the New York University Blood Center from three healthy donors and from blood samples of four vaccines enrolled in an AIDS study (3). PBMCs were purified with Ficoll-Hypaque and were subjected to RNA extraction with the RNA Easy kit (Qiagen, Santa Clarita, Calif.). The extracted RNA (4 \mu g) was then reverse transcribed with the Superscript II RT kit (Gibco BRL, Gaithersburg, Md.) and random hexamers according to the manufacturer’s recommendations. A nested PCR (nPCR) was initiated with the cDNA product and consensus external and internal sets of primers for the Ig heavy chain under the PCR conditions described previously (2). The final amplified nPCR products obtained from PBMCs of healthy donors were subsequently analyzed by ligating, cloning, and sequencing (Invitrogen Inc., Carlsbad, Calif.). The plasmid clones generated from the PBMCs of a healthy donor served as templates for the generation of probes for HTA. In this study, only two of the seven major VH human gene families were studied. Among the different VH human gene families, the frequency of use of these two gene families seems to be the highest (2).

The nPCR products were generated in a 50-\mu l reaction volume. The amplified products were first run on a gel to compare the intensities of the DNA bands; bands that exhibited equal intensities were analyzed by HTA. The comparison of bands not only confirmed successful cDNA synthesis and a successful amplification reaction but it also ensured that equal amounts of DNA were used in the assay. HTA was carried out essentially as described previously (11) by adding 1 \mu l of radiolabeled ss probe to 5 \mu l of the nPCR products in a total volume of 10 \mu l of annealing buffer (100 mM NaCl, 10 mM Tris [pH 7.4], 2 mM EDTA). Heteroduplexes were formed by melting the nPCR products at 94°C for 3 min in the presence of the probe and by cooling the mixture to 4°C in a thermocycler. The duplexes thus formed were then separated on a 5% polyacrylamide gel (acrylamide and bisacrylamide [30:1]) at 250 V for 2 h. Finally, the gels were dried under vacuum and were subjected to autoradiography.

ssDNA probes were generated by PCR with a 5' biotin-tagged variable-region heavy-chain primer and a 5' 32P-labeled JH primer. The JH primers were radiolabeled by use of the T4 kinase kit (Amersham International plc). The amplified product was purified with magnetic M280-streptavidin Dynal beads (Dynal Inc., Oslo, Norway). The bound product was eluted with 0.1 N NaOH, collected, and neutralized with 0.2 N HC1–1 M Tris-HCl (pH 8.0) in a total volume of 50 \mu l.

For the study of assay performance, nPCR products were amplified from the VH plasmid clones and were then analyzed by HTA with an ss probe. An ss VH 3-JH 1/4/5 probe was able to detect different duplexes generated from plasmids, and it could also distinguish the presence of two different clones in a reaction mixture. The two clones were identified as a homoduplex and a heteroduplex, respectively, on the basis of their mobilities (Fig. 1). When PBMCs were subjected to HTA with the same (VH 3-JH 1/4/5) probe, a smear was observed on the autoradiographs. The smear was an indication of poly-
clonality for $V_{H3}$ B cells, and it resulted from migration of Ig sequences to a separate location on the gel after they reannealed to a radiolabeled Ig ss probe. An occasional oligoclonality among the PBMC B cells was sometimes also identified as a prominent dark band among the polyclonal population (e.g., Fig. 1, lane 5).

Next, the specificity of the assay was evaluated by mixing a fixed number ($5 \times 10^2$, $5 \times 10^3$, $5 \times 10^4$, $5 \times 10^5$, $5 \times 10^6$, or 0) of monoclonal HL-6 B cells (with human $V_{H3}$-$J_{H4}$ genes; kindly provided by Marshall Posner) with PBMCs ($5 \times 10^6$). The cells were analyzed by HTA with an $ss$ $V_{H3}$-$J_{H1/4/5}$ probe. The arrow indicates the location of the clonal product for HL-6 cells within the PBMC population. The ratios at which HL-6 cells were mixed with PBMCs are indicated at the top.

In summary, I have described here for the first time a method that can be used to track clonal development among B cells in humans by HTA. The assay can find applications for the detection of B-cell lymphomas and plasmacytomas by recognizing them as predominant clonotypes or, in many cases, as the only clonotypes. It can also be used to study the immune response by observing changes in clonal types over time. The present assay design is limited in that it cannot distinguish between antigen-specific and nonspecific B cells if duplexes
generated from these cells were to band at an identical location. A modification of this technique with panned antigen-specific B cells may help in resolving this limitation of HTA.

Nucleotide sequence accession number. The sequences of plasmid clones (one clone for each V_H gene family) generated from the PBMCs of a healthy donor were submitted to the GenBank-National Center for Biotechnology Information database (accession no. AF129749-54).

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REFERENCES

1. Bakkus, M. H. 1999. Ig gene sequences in the study of clonality. Pathol. Biol. (Paris) 47:128–147.
2. Brezinschek, H. P., R. I. Brezinschek, and P. E. Lipsky. 1995. Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. J. Immunol. 155:190–202.
3. Connor, R. L., B. T. M. Korber, B. S. Graham, B. H. Hahn, D. D. Ho, B. D. Walker, A. U. Neumann, S. H. Vermund, J. Mestecky, S. Jackson, E. Fenmore, Y. Cao, F. Gao, S. Kalams, K. J. Kustman, D. McDonald, N. McWilliam, A. Trakola, J. P. Moore, and S. M. Wolinsky. 1998. Immunological and virological analyses of persons infected by HIV type 1 while participating in trials of recombinant gp 120 subunit vaccines. J. Virol. 72:1552–1576.
4. Delassus, S., A. Gey, S. Darche, A. Cumano, C. Roth, and P. Kourilsky. 1995. PCR-based analysis of the murine immunoglobulin heavy-chain repertoire. J. Immunol. Methods 184:219–229.
5. Gonzalez, M., D. Gonzalez, R. Lopez-Perez, R. Garcia-Saz, M. C. Chillon, A. Balanzategui, M. V. Mateos, I. Aloejo, A. W. Langerak, A. Orfao, J. J. Van Dongen, and M. F. San Migue. 1999. Heteroduplex analysis of vDJ amplified segments from rearranged IgH genes for clonality assessments in B-cell non-Hodgkin’s lymphoma. A comparison between different strategies Hematologica 84:779–784.
6. Greenberg, S. J., Y. Choi, M. Ballow, T. Du, P. M. Ward, M. H. Rickert, S. Frankel, S. H. Bernstein, and M. L. Brecher. 1995. Profile of immunoglobulin heavy chain variable chain variable gene repertoires and highly selective detection of malignant clonotypes in acute lymphoblastic leukemia J. Leukoc. Biol. 57:856–863.
7. Kohler, H., J. Goutsmit, and P. Nara. 1992. Clonal dominance: cause for a limited and failing immune response to HIV-1 infection and vaccination. J. Acquir. Immune Defic. Syndr. 5:1158–1168.
8. Kostense, S., F. M. Raaphorst, D. W. Notermans, J. Joling, B. Hooibrink, N. G. Pakker, S. A. Danner, J. M. Teale, and F. Miedema. 1998. Diversity of the T-cell receptor BV repertoire in HIV-1-infected patients reflects the biphasic CD4+ T-cell repopulation kinetics during highly active antiretroviral therapy. AIDS 12:F235–F240.
9. Lee, S. K., S. L. Bridges, P. M. Kirkham, W. H. Koopman, and H. W. Schroeder. 1994. Evidence of antigen receptor-influenced oligoclonal B lymphocyte expansion in the synovium of a patient with longstanding rheumatoid arthritis. J. Clin. Invest. 93:361–370.
10. Minegishi, Y., K. Akagi, K.-I. Nishikawa, H. Okawa, and J.-I. Yata. 1996. Analysis of the CDR3 region of the rearranged IgH chain genes in patients with severe combined immunodeficiency and severe lymphopenia. J. Immunol. 156:4666–4671.
11. Shen, D.-F., L. Doukhan, S. Kalams, and E. Delwart. 1998. High-resolution analysis of T-cell receptor B-chain repertoires using DNA heteroduplex tracking: generally stable, clonal CDS5 expansions in all healthy young adults. J. Immunol. Methods 215:113–121.
12. Wack, A., D. Montagna, P. Dellabona, and G. Casorati. 1996. An improved PCR-heteroduplex method permits high-sensitivity detection of clonal expansions in complex T cell populations. J. Immunol. Methods 196:181–192.