

REARRANGEMENT AND EXPRESSION OF T CELL  
RECEPTOR GENES IN CLONED MURINE NATURAL  
SUPPRESSOR CELL LINES

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Naturally occurring suppressor cells of the in vitro mixed leukocyte culture reaction and of in vivo graft-vs.-host disease have been identified in the spleens of neonatal mice (1) and of adult mice recovering from total lymphoid irradiation (2), whole-body irradiation (3), and syngeneic marrow transplantation (4), or cyclophosphamide therapy (5). Using both positive and negative selection procedures, the suppressors were reported to be null lymphocytes that did not express mature macrophage surface markers, nor differentiate into mature macrophages in vitro, nor demonstrate natural killer (NK) activity (1). Subsequently, cloned lines of these natural suppressor (NS) cells were derived from either adult mice given total lymphoid irradiation (TLI) (2) or from neonates (6). The cloned NS cell lines expressed a surface phenotype (2, 6) similar to that reported previously for cloned NK cells (Thy-1<sup>+</sup>, asialo-GM1<sup>+</sup>, Ig<sup>-</sup>, Lyt-1<sup>-</sup>, Lyt-2<sup>-</sup>, Ia<sup>-</sup>, MAC-1<sup>-</sup>) (7-9). However, the NS cells did not show NK activity in the standard assay with YAC-1 target cells. The cloned NS lines suppressed the proliferation of responder cells and the generation of cytolytic cells in the mixed leukocyte reaction (MLR), and suppressed lethal graft-vs.-host disease in vivo (10, 11). In view of the unusual function and surface phenotype of the cells, the lineage of these cells remained unclear.

To determine the lineage of the cloned NS cells, we searched for expression and rearrangement of the  $\alpha$  and  $\beta$  chain genes of the T cell antigen receptor, as well as that of the  $\gamma$  chain gene. Studies of the phenotypically similar NK cell yielded conflicting results. Thus, cloned lines of murine NK cells were reported to have rearrangements of the  $\beta$  chain genes, and to express mRNA for all three chains (12). In contrast, freshly purified rat or human large granular lymphocytes (LGL) were shown to express only the 1.0 kb mRNA species of the  $\beta$  chain gene (13), indicative of D-J joining (14). Thus, some but not all cells with NK function express the T cell receptor and are members of the T cell lineage. The current

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report shows that the NS lines express full-length mRNA transcripts for the  $\alpha$  and  $\beta$  chain of the T cell receptor, as well as the  $\gamma$  chain gene.

### Materials and Methods

*Cell Lines.* Three clones NS lines TLI-2.4C, 4BA4, and A1 were derived from the spleens of adult BALB/c mice treated with total lymphoid irradiation, neonatal BALB/c mice, and normal adult BALB/c mice using techniques described in detail previously (2, 6). These lines have been propagated continuously for at least 1 yr in tissue culture medium containing RPMI-1640 supplemented with 10 mM HEPES buffer, 2 mM glutamine (Gibco, Grand Island, NY),  $5 \times 10^{-5}$  M 2-ME (Sigma Chemical Co., St. Louis, MO), supernatants from Con A-stimulated rat spleen cells (10–20% vol/vol) and FCS (Hyclone, Logan, UT) (10% vol/vol).

*Immunofluorescence Staining of Cells.* A two-stage immunofluorescent staining procedure was used to detect surface markers on the NS cell lines as described previously (2, 6). Rat anti-mouse mAbs that were used in the first stage include anti-Thy-1.2 (clone 30-H12 obtained from Dr. I. L. Weissman, Dept. of Pathology, Stanford University), anti-L3T4 (clone GK.15 obtained from Dr. C. G. Fathman, Dept. of Medicine, Stanford University), anti-Lyt-1 (clone 53-7.3, Becton-Dickinson Immunocytometry Systems, Mountain View, CA), anti-Lyt-2 (clone 53-6.7, Becton-Dickinson Immunocytometry Systems), anti-IgM (clone 11B5 obtained from Dr. Weissman), and anti-MAC-1 (obtained from Dr. J. Monaco, Dept. of Medicine and Microbiology, Stanford University). A fluoresceinated goat anti-rat immunoglobulin antibody was used as the second stage (Caltag Laboratories, South San Francisco, CA). Analysis was performed using a fluorescence-activated cell sorter (FACS III, Becton-Dickinson Immunocytometry Systems) as in previous studies (2, 6). Background staining with an irrelevant rat anti-human idiotype mAb (obtained from Dr. R. Levy, Dept. of Medicine, Stanford University) was 2–3%.

*Northern Blot Analysis.* Cytoplasmic RNA was prepared (15) from NS cell lines TLI-2.4C, A1, and 4BA4. Northern blot hybridizations were performed (15, 16) using the following probes: T cell receptor  $\alpha$  chain: TT 11 cDNA clone (17); T cell receptor  $\beta$  chain: cDNA clone 86T5 (18);  $\gamma$  chain:  $\gamma$  cDNA clone (kindly provided by Dr. Ken-Ichi Arai; DNAX Research Institute of Molecular and Cellular Biology, Inc., Palo Alto, CA). The probes were labeled by the hexamer-labeling technique (19). Hybridization conditions were 50% formamide, 10% dextran sulfate, and  $5\times$  SSPE (saline sodium phosphate/EDTA buffer) (15) at 42°C for 16 h. Filters were washed twice for 30 min in  $2\times$  SSPE, 1% SDS at 55°C, and 30 min in  $0.2\times$  SSPE at 55°C.

*Southern Blot Analysis.* DNA from BALB/c liver cells, BALB/c NS cell lines, 4BA4, and TLI-2.4C were prepared by standard methods (15), digested with the Pvu II restriction endonuclease, electrophoresed through 0.7% agarose gels for 36 h and blotted onto nitrocellulose. Hybridization was performed using the  $\beta$  chain gene containing cDNA clone 86T5 (18). Hybridization and washing conditions were similar to those above.

### Results

*Function and Surface Markers of Three Cloned NS Cell Lines.* All three NS cell lines added to the MLR between normal adult BALB/c responder spleen cells ( $5 \times 10^5$ ) and irradiated (3,000 rad) C57BL/Ka stimulator spleen cells ( $5 \times 10^5$ ) suppressed peak [ $^3$ H]thymidine incorporation by at least 80% when the ratio of responder to NS cells was 10:1 (data not shown). In addition, the TLI-2.4C and 4BA4 cells ( $1.5 \times 10^7$ ) were able to suppress lethal graft-vs.-host disease in vivo when coinjected with  $5 \times 10^6$  C57BL/Ka spleen cells into sublethally irradiated weanling BALB/c mice such that >80% of recipients survived at least 100 d (11). <15% of recipients coinjected with a control BALB/c derived T cell line (HT-2) survived during the same time interval (11). The TLI-2.4C and 4BA4

TABLE I  
*Surface Markers Present on the TLI-2.4C, 4BA4, and A1 Lines  
 of NS Cells*

| Surface marker | Positively stained cells |
|----------------|--------------------------|
|                | %                        |
| Thy 1.2        | >90                      |
| L3T4           | <5                       |
| Lyt-1          | <5                       |
| Lyt-2          | <5                       |
| IgM            | <5                       |
| MAC-1          | <5                       |

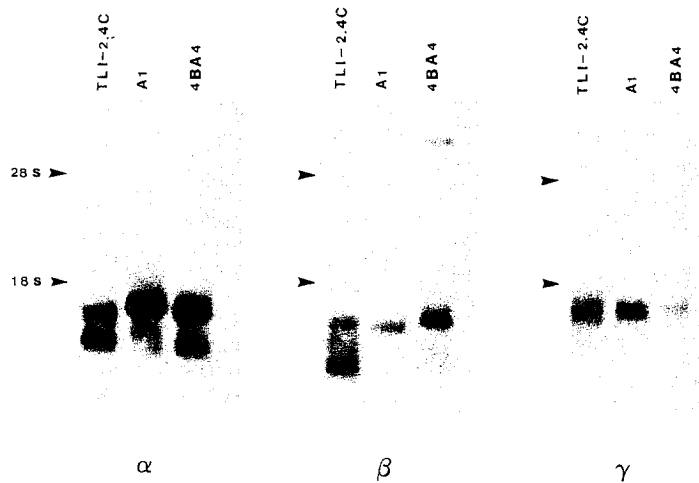


FIGURE 1. Northern blots of RNA extracted from the TLI-2.4C, A1, and 4BA4 NS cell lines. RNA was hybridized with cloned cDNA probes of the  $\alpha$ ,  $\beta$ , and  $\gamma$  chains of the T cell antigen receptor as described in Materials and Methods.

lines do not express NK function, since they fail to kill YAC-1 cells in vitro (2, 6).

Table I shows a representative example of the stable immunofluorescent staining pattern of the three cell lines. All were strongly Thy-1<sup>+</sup>, but showed minimal staining with antibodies directed to L3T4, Lyt-1, Lyt-2, IgM, and MAC-1 surface markers.

*Rearrangement and Expression of T Cell Receptor Genes in NS Lines.* The three cloned NS cell lines described above were used for the investigation of the T cell receptor expression of the  $\alpha$  and  $\beta$  chain, as well as that of the  $\gamma$  chain gene. The autoradiograph depicted in Fig. 1 shows that  $\alpha$ ,  $\beta$ , and  $\gamma$  chain mRNA of appropriate sizes were present in all NS lines. In the case of the  $\beta$  chain gene, the full-length mRNA of 1.3 kb, attributed to V-D-J rearrangements, was present in all cell lines. This indicated that the NS cells may assemble a functional T cell antigen receptor.

In addition, the NS cell lines TLI-2.4C and 4BA4 were examined for rearrangement of the  $\beta$  chain locus. Fig. 2 represents a Southern blot of Pvu II-digested DNA from the NS cell lines hybridized with a nick-translated insert

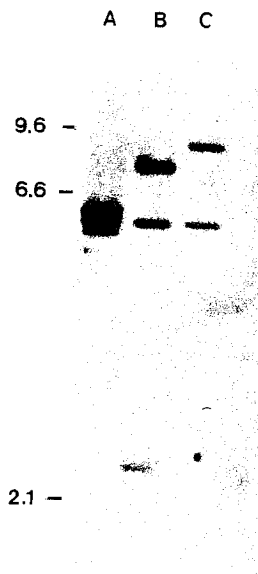


FIGURE 2. Southern blot of DNA extracted from BALB/c liver (A), from the TLI-2.4C cell line (B), and from the 4BA4 cell line (C). DNA was digested with Pvu II and hybridized with a cloned  $C_{\beta}$  cDNA probe. Molecular markers (kb) are shown on the left margin.

from 86T5, a  $C_{\beta}$  cDNA probe (20). DNA from BALB/c liver, TLI-2.4C, and 4BA4 cells was used. The two cell lines displayed one allele in the germline configuration. In addition, there were new (rearranged) bands distinct for the cell lines. Thus, NS cell lines of different origin showed clonal rearrangement of the  $\beta$  chain locus.

#### Discussion

The experiments reported herein demonstrated that natural suppressor cells were of the T cell lineage because of the  $\beta$  chain locus rearrangement and the expression of the  $\alpha$  and  $\beta$  chain genes of the T cell antigen receptor. The NS cells also expressed the  $\gamma$  chain gene. Immature, Lyt-2<sup>-</sup>, L3T4<sup>-</sup> thymocytes derived from fetal or adult mice express high levels of the  $\gamma$  chain mRNA (21, 22). However, unlike the NS cells, these cell populations did not express the  $\alpha$  chain gene. Mature cytotoxic T cell lines and hybridomas carrying the phenotype Lyt-2<sup>+</sup>, L3T4<sup>-</sup> also showed high-level expression of the  $\gamma$  chain gene (23, 24). The NS cells do not express the Lyt-2 surface marker and, therefore, do not belong to this group of cells. Finally, high  $\gamma$  chain gene expression was reported for Lyt-2<sup>-</sup>, L3T4<sup>+</sup> T cells activated by allogeneic stimuli in vitro (25) and for LGL lines with or without NK cell function (12). It is possible that the LGL lines without NK activity express NS function, and that some NK and NS cells are subsets of an LGL lineage that are derived from pre-T cells. Thus, members of the T cell lineage with NS and/or NK cell function(s) may represent parallel lines of nonspecific suppressor and cytolytic cell development similar to that of the antigen-specific Ts and Tc cell development.

In conclusion, cloned NS cells are members of the T cell lineage that express the  $\alpha$ ,  $\beta$ , and  $\gamma$  chain genes. Similar studies are necessary to determine whether freshly purified NS cells also express these genes.

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### References

1. Oseroff, A., S. Okada, and S. Strober. 1984. Natural suppressor (NS) cells found in the spleen of neonatal mice and adult mice given total lymphoid irradiation (TLI) express the null surface phenotype. *J. Immunol.* 132:101.
2. Hertel-Wulff, B., S. Okada, A. Oseroff, and S. Strober. 1984. In vitro propagation and cloning of murine natural suppressor (NS) cells. *J. Immunol.* 133:2791.
3. Waer, M., K. K. Ang, E. van der Schueren, and M. Vandeputte. 1984. Allogeneic bone marrow transplantation in mice after total lymphoid irradiation: influence of breeding conditions and strain of recipient mice. *J. Immunol.* 132:991.
4. Waer, M., K. K. Ang, E. van der Schueren, and M. Vandeputte. 1984. Increased incidence of murine graft versus host disease after allogeneic bone marrow transplantation by previous infusion of syngeneic bone marrow cells. *Transplantation (Baltimore)*. 38:396.
5. Segre, M., E. Tomei, and D. Segre. 1985. Cyclophosphamide-induced suppressor cells in mice: Suppression of the antibody responses in vitro and characterization of the effector cells. *Cell. Immunol.* 91:443.
6. Schwadron, R. B., D. M. Gandour, and S. Strober. 1985. Cloned natural suppressor cell lines derived from the spleen of neonatal mice. *J. Exp. Med.* 162:297.
7. Dennert, G., G. Yogeewaren, and S. Yamagata. 1981. Cloned cell lines with natural killer activity. Specificity, function, and cell surface markers. *J. Exp. Med.* 153:545.
8. Nabel, G., L. R. Bucalo, J. Allard, H. Wigzell, and H. Cantor. 1981. Multiple activities of a cloned cell line mediating natural killer cell function. *J. Exp. Med.* 153:1582.
9. Brooks, C. G., K. Kuribayashi, G. E. Sale, and C. S. Henney. 1982. Characterization of five cloned murine cell lines showing high cytolytic activity against YAC-1 cells. *J. Immunol.* 128:2326.
10. Schwadron, R. B., and S. Strober. 1987. Cloned natural suppressor (NS) cells derived from the neonatal spleen: In vitro action and lineage. *Transplant. Proc.* 19:533.
11. Hertel-Wulff, B., V. Palathumpat, R. Schwadron, and S. Strober. 1987. Prevention of graft versus host disease by natural suppressor cells. *Transplant. Proc.* 19:536.
12. Ikuta, K., M. Hattori, K. Wake, S. Kano, T. Honjo, J. Yodoi, and N. Minato. 1986. Expression and rearrangement of the  $\alpha$ ,  $\beta$ , and  $\gamma$  chain genes of the T cell receptor in cloned murine large granular lymphocyte lines: No correlation with the cytotoxic spectrum. *J. Exp. Med.* 164:428.
13. Young, H. A., J. R. Ortaldo, R. B. Herberman, and C. W. Reynolds. 1986. Analysis of T cell receptors in highly purified rat and human large granular lymphocytes (LGL): Lack of functional 1.3 kb  $\beta$ -chain mRNA. *J. Immunol.* 136:2701.
14. Kavalier, J., M. M. Davis, and Y. Chien. 1984. Localization of a T-cell receptor diversity-region element. *Nature (Lond.)*. 310:421.
15. Maniatis, T., E. F. Fritsch, and J. Sambrook. In *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.
16. Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641.
17. Chien, Y-H., D. M. Becker, T. Lindsten, M. Okamura, D. I. Cohen, and M. M. Davis. 1984. A third type of murine T-cell receptor gene. *Nature (Lond.)*. 312:31.
18. Hedrick, S. M., D. I. Cohen, E. A. Nielsen, and M. M. Davis. 1984. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature (Lond.)*. 308:149.

19. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6.
20. Hedrick, S. M., E. A. Nielsen, J. Kavaler, D. I. Cohen, and M. M. Davis. 1984. Sequence relationships between putative T-cell receptor polypeptides and immunoglobulins. *Nature (Lond.)*. 308:153.
21. Raulat, D. H., R. D. Garman, H. Saito, and S. Tonegawa. 1985. Developmental regulation in T-cell receptor gene expression. *Nature (Lond.)*. 314:103.
22. Samelson, L. E., T. Lindsten, B. J. Fowlkes, P. van den Elsen, C. Terhorst, M. M. Davis, R. N. Germain, and R. H. Schwartz. 1985. Expression of genes of the T-cell antigen receptor complex in precursor thymocytes. *Nature (Lond.)*. 315:765.
23. Saito, H., D. M. Kranz, Y. Takagaki, A. C. Hayday, N. H. Eisen, and S. Tonegawa. 1984. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature (Lond.)*. 309:757.
24. Kranz, D. M., H. Saito, M. Heller, Y. Takagaki, W. Haas, H. N. Eisen, and S. Tonegawa. 1985. Limited diversity of the rearranged T-cell gamma genes. *Nature (Lond.)*. 313:752.
25. Jones, B., S. Mjolsness, C. Janeway, Jr., and A. C. Hayday. 1986. Transcripts of functionally rearranged gamma genes in primary T cells in adult immunocompetent mice. *Nature (Lond.)*. 323:635.