Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 4508–4512, July 1985
Immunology

Generation and regulation of autocytotoxicity in mixed lymphocyte cultures: Evidence for active suppression of autocytotoxic cells

(autoimmunity/immunologic tolerance/limiting dilution analysis/graft-versus-host disease/immunoregulation)

KAREN ROSENKRANTZ*, BO DUPONT, AND NEAL FLOMENBERG

Human Immunogenetics and Effector Lymphocyte Biology Laboratories, Memorial Sloan–Kettering Cancer Center, 1250 First Avenue, New York, NY 10021

Communicated by Edward A. Boyse, March 20, 1985

ABSTRACT **Using limiting dilution analysis, we have detected both the generation and suppression of autocytotoxic cells following autologous or allogeneic stimulation *in vitro*. Assay conditions were established in which the cytotoxic response toward an allogeneic sensitizing cell was consistent with a traditional single-hit kinetic model. Under identical conditions, cytolytic activity toward autologous phytohemagglutinin-activated lymphoblasts exhibited a distinct biphasic response. At low responder cell doses, a clear autocytotoxic response was observed. However, at higher responder cell numbers, this autocytotoxic reaction disappeared. This biphasic pattern of autocytotoxicity developed after stimulation with allogeneic or autologous peripheral blood mononuclear leukocytes (PBL) or Epstein–Barr virus-transformed B cells. This pattern of response is consistent with the counterpoised actions of two distinct cell populations, an autoaggressive population and a lower frequency autosuppressor population. Autocytotoxicity was not the result of mitogenic or xenogeneic antigenic stimulation, as it was observed after stimulation with autologous PBL in autologous serum and an autologous interleukin 2 preparation. Thus, cells capable of autocytotoxicity are present in peripheral blood but at a lower frequency than allocytotoxic T lymphocytes. Furthermore, autoaggressive cells are downregulated by an autologous suppressor population. These findings suggest that immunologic self-tolerance is, at least in part, an actively maintained condition. Disturbances in this autoregulatory network may have relevance to the pathogenesis of some autoimmune diseases and graft-versus-host disease.**

One of the most striking characteristics of the immune system is its ability to react with a broad variety of foreign antigens while being selectively tolerant to "self" (1, 2). The T-cell repertoire and the capacity for the recognition and tolerance of self appear to be critically shaped in the thymus as T cells develop immunocompetence (3–5). The mechanisms by which immunologic autoaggression is prevented remain unknown. While several models have been proposed, they suggest, in general, either the clonal elimination of self-aggressive cells during T-cell ontogeny (2) or the ongoing suppression of such cells (reviewed in ref. 6).

Peripheral blood mononuclear leukocytes (PBL) do not in general proliferate or develop cytotoxicity *in vitro* when exposed to autologous cells. Purified T cells, however, do proliferate in response to autologous non-T cells in the syngeneic or autologous mixed lymphocyte culture reaction (7, 8). Although interleukin 2 (IL-2) production and T-cell proliferation are regularly seen in the autologous mixed lymphocyte culture (9), the generation of autocytotoxic T lymphocytes is an inconsistent finding (10, 11). An autoaggressive response has occasionally been demonstrable when the responder PBL population is fractionated in some way (12, 13) or when exogenous growth factors are added (14). Autoaggressive cells have similarly been isolated from the blood of patients with aplastic anemia (15, 16). These findings together suggest that autocytotoxic cells can be generated, but only under conditions in which a homeostatic regulatory system present within normal unfractionated PBL has been disturbed. Recent studies in rats suggest that normal but not irradiated animals possess a regulatory mechanism capable of controlling autoaggressive T cells (17).

By using limiting dilution analysis (LDA), the present studies demonstrate that an autoaggressive response is reproducibly generated after *in vitro* stimulation with autologous or allogeneic cells. It is further demonstrated that, in contrast to allocytotoxic cells, autoaggressive cells exhibit a biphasic pattern of response and are normally downregulated by a second PBL population.

MATERIALS AND METHODS

Cell Populations. PBL were isolated from healthy human donors by Ficoll-sodium metrizoate density gradient centrifugation (18) (Lymphoprep, Accurate Chemicals, Westbury, NY). B-lymphoblastoid cell lines (BLCL) were generated with Epstein–Barr virus as described (19).

IL-2 Source. Three sources of IL-2 were used as follows: (*i*) IL-2 containing human conditioned medium (IL-2/HC medium) was produced from PBL that had been pulsed for 2 hr with purified phytohemagglutinin (PHA-HA-16, Burroughs Wellcome, Research Triangle Park, NC) and phorbol myristic acetate (Sigma) as described (20). (*ii*) MLA-IL-2 from the MLA-144 cell line (21) (American Type Culture Collection) was produced in the same fashion as IL-2/HC medium except that cells were pulsed only with phorbol myristic acetate. (*iii*) Recombinant IL-2 (Amgen, Thousand Oaks, CA) was used at a final concentration of 25 units/ml.

LDA Assay. LDA assays were performed in V-bottom Linbro microtiter plates (Flow Laboratories) in 0.2 ml of supplemented RPMI 1640 medium (GIBCO) with 10% autologous (responder) serum and an IL-2 source. Wells contained graded concentrations of responder PBL and 5×10^4 irradiated stimulator PBL (4000 rads; 1 rad = 1.0×10^{-2} gray) or BLCL (8000 rads). On the 7th day, 100 μl of medium was removed and replaced with 100 μl of supplemented RPMI 1640 medium with 10% pooled human serum or autologous serum and IL-2. On the 10th day, individual wells were assayed for cytotoxicity in a chromium release assay by adding 3×10^3 ^{51}Cr-labeled (5 mCi/ml; 1 Ci = 37 GBq; New England Nuclear) target cells to each well (22). The supernatants were harvested after 4 hr. PHA-activated lympho-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: BLCL, B-lymphoblastoid cell line(s); IL-2, interleukin 2; LDA, limiting dilution analysis; PBL, peripheral blood mononuclear leukocyte(s); PHA, phytohemagglutinin.

*On leave of absence from the Department of Surgery, College of Physicians and Surgeons, Columbia University, New York, NY.

blast target cells were prepared by culturing PBL in 1% PHA-M (Difco) for 4 days.

The cytolytic activity of an individual well was scored as positive when the release of ^{51}Cr exceeded the mean of the control plate (stimulator cells alone) by 3 SD. The Rankit test (23) was used to demonstrate that the counts obtained from a set of eight control plates (stimulators alone) were distributed normally.

Statistical Analysis. Kinetic analysis and precursor frequency determinations were performed by the statistical methods of χ^2 minimization as described by Taswell (24). When the data showed $P > 0.05$, the relationship between responder cell dose and the number of nonresponding wells (F_0) was considered consistent with a single-hit kinetic model. The precursor frequency (f) is equivalent to the slope of the regression line.

In the analysis and quantitation of suppressor cells, the zero-order term of the Poisson equation instead reflects those cultures that respond (i.e., do not contain a suppressor cell) (25). The relationship between responder cell dose and the number of responding test wells (F_+) was analyzed as described above.

RESULTS

LDA of the Allocytotoxic Response. Data from a LDA of a representative allogeneic priming between two unrelated individuals is shown in Fig. 1. The frequency of allocytotoxic cells was 1/1723. When 10,000 irradiated responder cells were added to each well, the frequency estimate increased slightly to 1/733. The reaction kinetics were in each case consistent with a single-hit model. Therefore, to provide maximal allocytotoxic precursor frequencies (f), each well was routinely supplemented with 10,000 irradiated responder cells. The range of f for allocytotoxic cells varied between 1/119 and 1/3018, depending on the specific responder cells, stimulator cells, and the batch of IL-2 used. In all allogeneic sensitizations, the cytolytic response remained strongly positive in all test wells at responder cell doses >10,000.

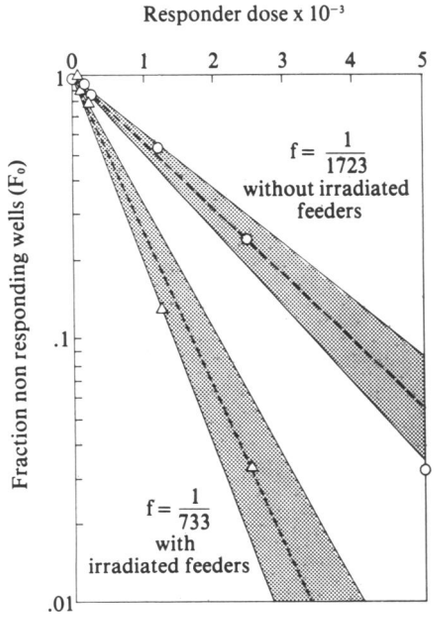

FIG. 1. LDA of the allocytotoxic response. Increasing numbers of responder RWK PBL (HLA-A2, w33, B7, w35, w6, Cw7, DR2, -6) were stimulated by KR PBL (HLA-A1, B8, w6, Cw7, DR3, -4) in 10% RWK serum and 15% IL-2 HC medium with (△) and without (○) 10,000 irradiated RWK PBL per well; 96 wells were plated at each responder dose. Cytotoxicity was measured against KR PHA lymphoblasts; 10×10^3 and 50×10^3 responder dose points are not shown, as all wells were positive. In the absence of additional irradiated responder cells, $f = 1/1723$ ($P > 0.05$). In the presence of additional irradiated responder cells, $f = 1/733$ ($P > 0.05$).

Autocytotoxic Response After Stimulation with Autologous PBL or B-Lymphoblastoid Cell Lines (BLCL). The LDA techniques developed for enumeration of allogeneic cytotoxic T-lymphocyte precursors were then applied to autologous cell combinations. Autologous serum was used to minimize T-cell stimulation by foreign serum proteins. Surprisingly, in all individuals tested ($n = 14$), when irradiated autologous PBL or BLCL were used as stimulator cells, a strong autocytotoxic reaction against autologous PHA-stimulated lymphoblasts was invariably generated (Fig. 2). At low responder numbers, the number of wells demonstrating autocytolytic activity increased as the responder dose increased. Indeed, at optimal responder cell doses, 80–90% of the wells were positive. As the number of responder cells was increased further, however, the autocytolytic activity disappeared. Thus, in sharp contrast to the allocytotoxic response, the autocytotoxic reaction was biphasic. This biphasic pattern can be explained by the action of two counterpoised cell populations. The first population, present at higher frequency, is autocytotoxic. The second population, present at a lower frequency, inhibits the generation of autocytotoxicity. At low responder cell numbers, few wells receive the regulatory population and therefore an autoaggressive response can be observed. At higher responder cell doses, more wells receive regulatory cells, and the autoaggressive response disappears.

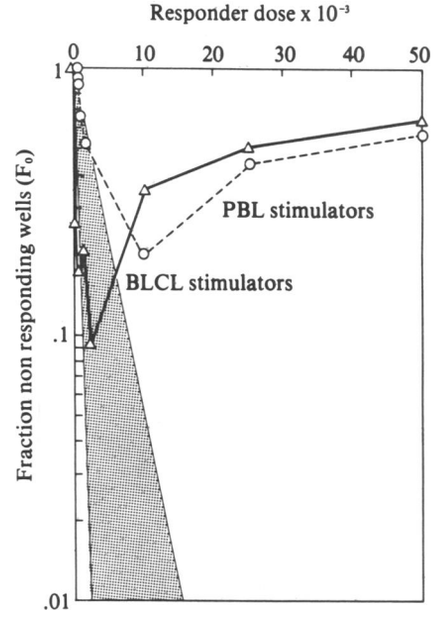

FIG. 2. LDA of the autocytotoxic response. NF PBL (HLA-A26, 28, B14, w41, w6, Cw8, DR1, -3) were stimulated by irradiated autologous PBL (○) or BLCL (△) in 10% NF serum and 10% MLA IL-2; 48 wells were plated at each responder dose. Cytotoxicity was measured against NF PHA lymphoblasts. Shaded area represents the experimental range of allospecific CTL precursor frequencies. When PBL were used as stimulator cells, positive wells at the point of inflection (responder cell dose = 10,000 cells per well) exhibited a mean percentage cytotoxicity of 12.0% with a range of 5.9% to 35.8%. On the control plate (stimulator cells alone), the mean chromium release + 3 SD corresponded to 5.5% cytotoxicity. When BLCL were used as stimulator cells, positive wells at the point of inflection (responder cell dose = 2000 cells per well) exhibited a mean percentage cytotoxicity of 15.4% with a range of 5.3% to 32.1%. The mean chromium release + 3 SD for the control plate corresponded to 5.3%.

The data from the descending portion of the autologous LDA graph (responder cell doses from zero to the point of inflection) were analyzed in semilogarithmic F_0 plots. When PBL were used as stimulators, F_0 plots were usually consistent with single-hit kinetics (Fig. 3*A*). Precursor frequencies for these autoreactive cells ranged from 1/2858 to 1/7899. The data from the ascending portion of the autologous LDA graph (responder cell doses from the point of inflection to 50,000) were analyzed in F_+ plots. When PBL were used as stimulators (Fig. 3*C*), data points on F_+ plots fell within the calculated 95% confidence intervals. This was consistent with an inhibitory cell inactivating autocytolytic cells in a single-hit fashion. The inhibitory cell precursor frequency range was 1/16,848 to 1/52,629.

Additional experiments were performed with autologous BLCL as stimulator cells. Autocytolytic activity was again measured using autologous PHA-activated lymphoblasts. The response patterns were biphasic, indicating the development of both autocytotoxic and autosuppressor cells (Fig. 2). However, neither the F_0 plots nor the F_+ plots conformed to a single-hit kinetic model after autologous BLCL stimulation (Fig. 3 *B* and *D*). The use of BLCL stimulator cells qualitatively changed the kinetic patterns of the response. Most likely, the BLCLs provide additional stimulating antigens, which are not expressed on PBL. Therefore, all subsequent experiments were performed with PBL stimulator cells.

To assess whether autoaggressive response was due to nonspecific lectin-dependent cellular cytotoxicity, autologous PHA lymphoblasts and autologous BLCL were compared as target cells (Fig. 4). After sensitization with autologous PBL stimulator cells, cytolytic activity against these two targets followed very similar biphasic curves. This suggested that the autocytolytic response was not simply a consequence of the presence of PHA in the test wells.

Additional LDAs with autologous PBL stimulator cells were performed in which the cytotoxicity against autologous PHA lymphoblasts was compared to that against a natural killer target, K562. Cells capable of lysing the K562 target were far more frequent than cells capable of autocytotoxicity. Moreover, while the autocytotoxic response remained biphasic, the K562 response was linear and remained strongly positive at increasing responder cell doses. Thus, the autoaggressive response differed both qualitatively and quantitatively from the natural killer cell activity generated in these cultures.

Finally, although all LDAs were performed using autologous serum, it remained possible, although unlikely, that the autocytolytic response was the result of stimulation by exogenous proteins in the lymphocyte-conditioned medium. A LDA was therefore performed in an entirely autologous system. Autologous PBL stimulator cells and an autologous IL-2/HC medium previously prepared from the responder's PBL in autologous serum were used for this culture. The same biphasic response was seen (Fig. 5*A*). The F_0 yielded a precursor estimate of 1/2864 for the autoaggressive population, while the F_+ yielded an estimate of 1/16,848 for the regulatory population (Fig. 5 *B* and *C*).

Autocytotoxic Response After Allogeneic Stimulation. The autocytotoxic and autosuppressive responses were analyzed after allogeneic sensitization. A biphasic response toward autologous PHA lymphoblasts was again seen (Fig. 6*A*). F_0 plots demonstrated single-hit kinetics and yielded an autocytotoxic precursor frequency of 1/4708 compared to an allocytotoxic precursor frequency of 1/478 (Fig. 6*B*). The autosuppressor frequency was sharply increased after allogeneic stimulation (Fig. 6*C*). Similar biphasic curves were obtained when allogeneic BLCL were used as stimulator cells (data not shown). Thus, the autoaggressive cell frequency

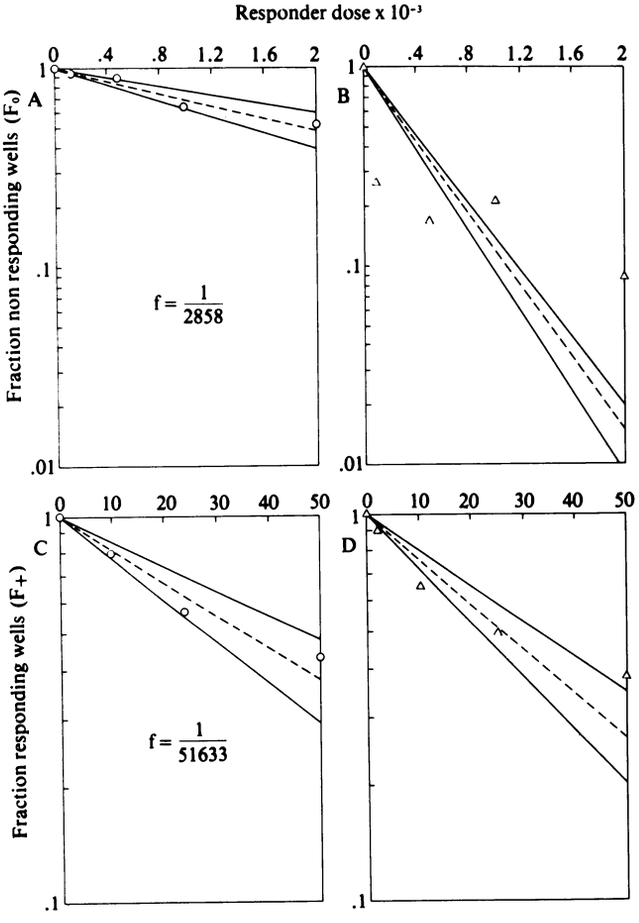

FIG. 3. Kinetic analysis of the autocytotoxic response. (*A*) F_0 plot of autologous PBL stimulation [f = 1/2858 ($P > 0.05$)]. (*B*) F_0 plot of autologous BLCL stimulation ($P < 0.01$). (*C*) F_+ plot of autologous PBL stimulation [f = 1/51,633 ($P > 0.05$)]. (*D*) F_+ plot of autologous BLCL stimulation ($P < 0.05$).

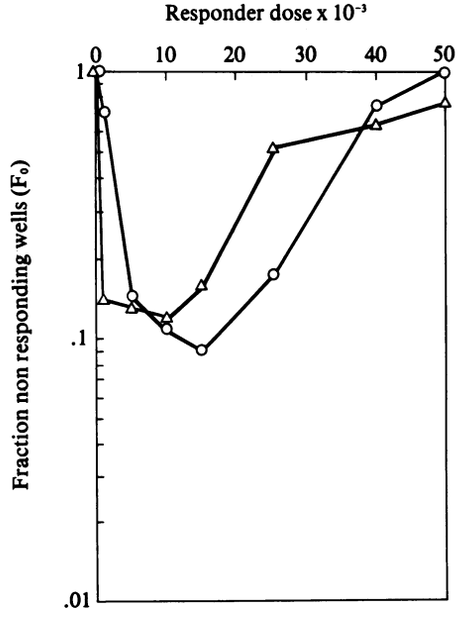

FIG. 4. Comparison of B-lymphoblastoid cell lines and PHA lymphoblasts as targets for autocytotoxic cells. KR PBL (HLA-A1, B8, Bw6, Cw7, DR3, -4) were stimulated with autologous PBL in 10% KR serum and 15% IL-2 HC medium; 96 wells were plated at each responder dose. Cytotoxicity was measured against autologous PHA lymphoblasts (○) and autologous BLCL (△).

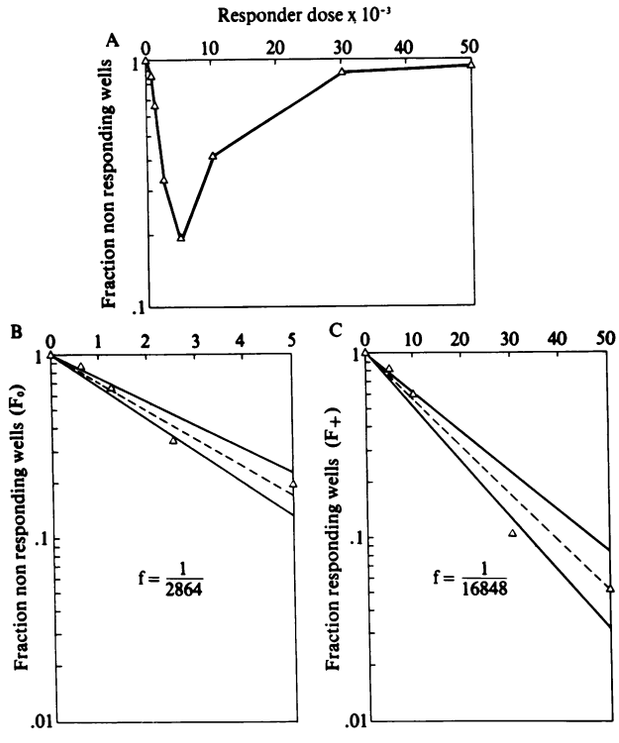

FIG. 5. Autocytotoxicity is generated using autologous HC medium. (*A*) NF PBL (HLA-A26, 28, B14, w41, w6, Cw8, DR1, -3) were stimulated with irradiated autologous PBL in 10% NF serum and 15% autologous IL-2 HC medium; 96 wells were plated at each responder dose. Cytotoxicity was measured against autologous PHA lymphoblasts. (*B*) F_0 plot of *A* [f = 1/2784 ($P > 0.05$)]. (*C*) F_+ plot of *A* [f = 1/16,848 ($P > 0.05$)].

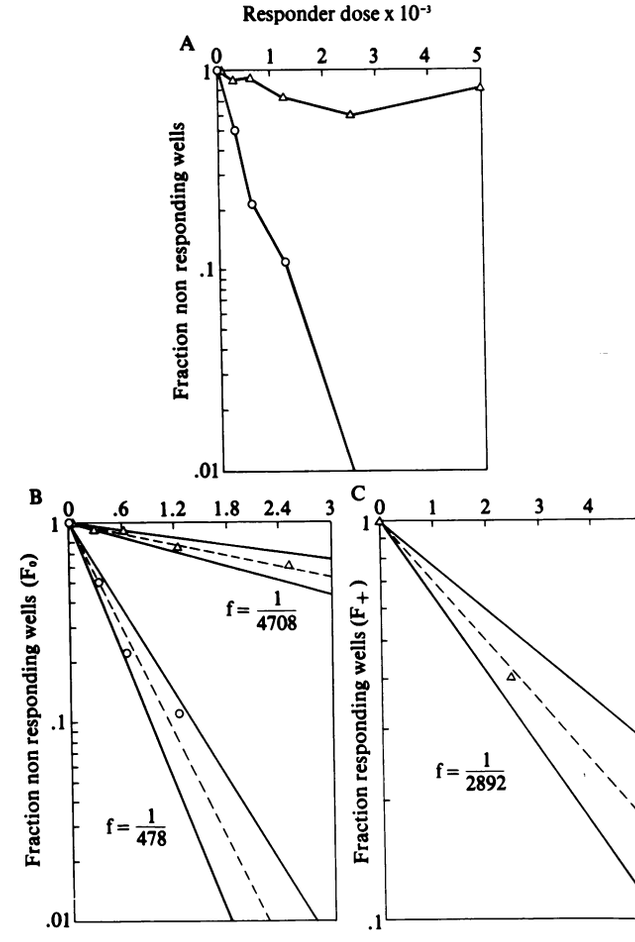

FIG. 6. (*A*) Autocytotoxic response after allogeneic stimulation. BJ PBL (HLA-A3, w30, Bw35, w60, w6, Cw3, w4, DR4, w6) were stimulated with irradiated DW PBL (A2, Bw41, w4, w6, Cw2, DR3) in the presence of 10,000 irradiated BJ PBL per well in 10% BJ serum and 10% MLA IL-2; 48 wells were tested at each responder cell dose against PHA lymphoblasts from BJ (△) and 48 additional wells were tested against PHA lymphoblasts from DW (○). (*B*) F_0 plot of autocytotoxic response toward autologous (△) BJ lymphoblasts: f = 1/4708 ($P > 0.05$); and allogeneic (○) DW lymphoblasts: f = 1/478 ($P > 0.05$). (*C*) F_+ plot of autocytotoxic response against BJ lymphoblasts: f = 1/2892 ($P > 0.05$).

after allogeneic stimulation is similar to that seen after autologous stimulation. In contrast, the frequency of regulatory cells is markedly increased.

DISCUSSION

The data presented in this report suggest that self-tolerance is maintained, in part, by an ongoing suppression of cells capable of autoaggression. Through the use of LDA, one sees, in effect, the breaking of self-tolerance at low responder cell doses and its subsequent reestablishment as the number of responder cells is progressively increased.

The possibility that the autoaggressive response was induced by a foreign protein or mitogen has been ruled out. Autocytotoxicity was observed even when the assays were performed in autologous serum and an autologous IL-2 preparation. Autocytotoxicity is detected in cultures with MLA IL-2, which has no PHA, and in cultures with recombinant IL-2, which has no PHA or phorbol myristic acetate (unpublished observations). Furthermore, the lysis of autologous BLCL targets ruled out a nonspecific lectin-dependent cytotoxicity of target PHA lymphoblasts.

Autocytotoxicity has previously been detected in LDAs when F1 mice were stimulated with parental cells (13). No decrease in the autocytotoxic reaction was noted. However, only data from low responder/stimulator ratios were reported. These would correspond to data points from the cytotoxic aspect of the biphasic curve in the present study. Both nonspecific natural killer-like (11, 12) and specific (10, 14, 16) autocytotoxic cells have been described.

The present studies demonstrate the existence of a regulatory population that suppresses the generation of autocytotoxic but not allocytotoxic cells. Similar biphasic curves have been reported in analyses of mixtures of cells that help and suppress immunoglobulin synthesis (26, 27). It is unclear whether autoaggressive cells are inhibited through direct cell-to-cell contact (either cytotoxic or noncytotoxic) or through the release of soluble factors by the regulatory population. It is unlikely that the disappearance of autocytotoxicity is due to exhaustion of the culture medium or overgrowth, as allocytolytic and natural killer activity remained strongly positive under the same culture conditions. In addition, suppression frequently appeared rapidly over a narrow range of responder cell doses. This suggested that it resulted from a highly active and specific mechanism rather than from nonspecific cold target cell competition from the expanding responder cell populations.

The present findings argue that self-tolerance is an actively maintained state, requiring the proper balance between autocytotoxic and autosuppressor cells to prevent the development of overt autoaggression. A lack of suppressor activity has been demonstrated in association with the development of autoimmunity in NZB mice (28). Furthermore, a disturbance in the autoregulatory balance may be associated with graft-versus-host disease following HLA identical or syngeneic bone marrow transplantation. A syndrome identical to graft-versus-host disease can be generated in rats after

withdrawal of cyclosporin A in syngeneic bone marrow transplant recipients (17). This syndrome can be adoptively transferred via infusion of T cells into irradiated but not normal animals. These findings are consistent with the presence of cells capable of down regulating autoaggressive T lymphocytes in the intact animals.

A second point emerges from the present studies concerning the mechanisms that maintain self-tolerance. Although cells capable of autoaggression do exist in normal peripheral blood, these cells exist at 1/10th the frequency of alloreactive cells. This is similar to the ratio of alloproliferative and autoproliferative cells reported previously (29). It must be emphasized that this comparison does not reflect the number of T cells responding to the entire spectrum of alloantigens, but rather the frequency of T lymphocytes that can respond to the alloantigens expressed by a single unrelated individual. It has previously been demonstrated that the frequency of alloreactive T cells does not increase after birth (30). Thus, the autoreactive population appears to have been selectively contracted compared to the alloreactive population, implying that the T-cell repertoire has been shaped by the clonal elimination of some but not all autoaggressive cells. This would suggest that self-tolerance is maintained by several mechanisms rather than by a single process. In addition to the clonal elimination of autoaggressive cells during T-cell ontogeny, the ongoing activity of an autoregulatory population appears essential for the maintenance of self-tolerance and the prevention of autoimmunity.

We thank Richard O'Reilly for his long-standing support and helpful discussions concerning this work, Keith Reemtsma for his continued help and support, Robert W. Knowles and Stuart MacPhail for helpful discussions, Carol Bodenheimer for help with statistical analyses, Donna Williams for excellent technical assistance, and Louise Rozos for help in preparing the manuscript. This work was supported by grants from the U.S. Public Health Services, National Institutes of Health, NCI-CA-22507, CA-08748, CA-19267, CA-23766, and CA-33050. K.R. was supported by the Rubinstein–Jacobs Fellowship for Oncological Surgery. N.F. was supported by a Clinical Scholars Award from the Norman and Rosita Winston Foundation.

1. Jerne, N. (1955) *Proc. Natl. Acad. Sci. USA* **41,** 849–857.
2. Burnet, F. M. (1959) *The Clonal Selection Theory of Acquired Immunity* (Cambridge University Press, Cambridge, England).
3. Miller, J. F. A. P. (1961) *Lancet* **ii,** 748–749.
4. Good, R. A., Dalmasso, A. P., Martinez, C., Archer, O. K., Pierce, J. C. & Papermaster, B. W. (1962) *J. Exp. Med.* **116,** 773–796.
5. Zinkernagel, R. M. & Doherty, P. C. (1974) *Nature (London)* **248,** 701–702.
6. Nossal, G. J. V. (1983) *Annu. Rev. Immunol.* **1,** 33–62.
7. Howe, M. L., Goldstein, A. L. & Battisto, J. R. (1970) *Proc. Natl. Acad. Sci. USA* **67,** 613–619.
8. Opelz, G., Kiuchi, M., Takasugi, M. & Terasaki, P. I. (1975) *J. Exp. Med.* **142,** 1327–1333.
9. Lattime, E. C., Gillis, S., Pecoraro, G. & Stutman, O. (1982) *J. Immunol.* **128,** 480–485.
10. Miller, R. A. & Kaplan, H. S. (1978) *J. Immunol.* **121,** 2165–2167.
11. Tomonari, K. (1980) *J. Immunol.* **124,** 1111–1121.
12. Parkman, R. & Rosen, F. S. (1976) *J. Exp. Med.* **144,** 1520–1530.
13. Reimann, J. & Miller, R. G. (1983) *J. Immunol.* **131,** 2128–2134.
14. Altman, A. & Katz, D. H. (1980) *J. Immunol.* **125,** 1536–1543.
15. Warren, R. P., Storb, R., Thomas, E. D., Su, P. J., Mickelson, E. M. & Weiden, P. L. (1980) *Blood* **56,** 683–689.
16. Mickelson, E. M., Beatty, P. G., Storb, R. & Hansen, J. A. (1984) *Hum. Immunol.* **10,** 189–201.
17. Glazier, A., Tutschka, P. J., Farmer, E. R. & Santos, G. W. (1983) *J. Exp. Med.* **158,** 1–8.
18. Boyum, A. (1968) *Scand. J. Clin. Invest. Suppl. 97* **21,** 77–89.
19. Hansen, J. A., Fu, S. M., Antonelli, P., Kamoun, M., Hurley, J. N., Winchester, R. J., Dupont, B. & Kunkel, H. G. (1979) *Immunogenetics* **8,** 51–64.
20. Meuer, S. C., Schlossman, S. F. & Reinerz, E. L. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4395–4399.
21. Rabin, H., Hopkins, R. F., Ruscetti, F. W., Neubauer, R. H., Brown, R. L. & Kawakami, T. G. (1981) *J. Immunol.* **127,** 1852–1856.
22. Schendel, D. J., Wank, R. & Dupont, B. (1979) *Tissue Antigens* **13,** 112–120.
23. Bliss, C. (1967) *Statistics in Biology* (McGraw-Hill, New York), pp. 108–112.
24. Taswell, C. (1981) *J. Immunol.* **126,** 1614–1619.
25. Lefkovits, I. & Waldmann, H. (1979) *Limiting Dilution Analysis of Cells of the Immune System* (Cambridge University Press, Cambridge, England).
26. Corley, R. B., Kindred, B. & Lefkovits, I. (1978) *J. Immunol.* **121,** 1082–1089.
27. Eichmann, K., Falk, I., Melchers, I. & Simon, M. M. (1980) *J. Exp. Med.* **152,** 477–492.
28. Allison, A. C., Denman, A. M. & Barnes, R. D. (1971) *Lancet* **ii,** 135–140.
29. Kozak, R. W., Moody, C. E., Staiano-Cocico, L. & Weksler, M. E. (1982) *J. Immunol.* **126,** 1723–1727.
30. Hayward, A. R. & Malmberg, S. (1984) *Pediatr. Res.* **18,** 414–419.