CLONED NATURAL SUPPRESSOR CELL LINES DERIVED FROM THE SPLEENS OF NEONATAL MICE

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Spleen cells of neonatal mice are capable of suppressing a variety of immune functions (1–12). Several laboratories have shown that neonatal spleen cells can suppress the mixed leukocyte reaction (MLR)[1] between normal adult responder cells and allogeneic stimulator cells, regardless of the haplotype of the responder or stimulator cells (1–7). The suppression of a variety of different in vivo assays measuring graft vs. host disease (GVHD) has also been demonstrated (8–10). Similar types of nonspecific suppressor cells have been described in animals treated with total lymphoid irradiation (TLI) (13), in early allogeneic radiation chimeras (14), and in irradiated animals repopulated with syngeneic bone marrow (15). Recent studies (16) suggest that these naturally occurring nonspecific suppressor cells may regulate the generation of antigen-specific suppressor cells, which maintain tolerance to foreign allografts.

Although the presence of nonspecific suppressor cells in the spleen of neonates has been generally accepted, the surface phenotype has been debated. Argyris (1, 2) reported that treatment of neonatal spleen cells with anti-Thy-1.2 antiserum and complement abrogated the ability of neonatal cells to suppress the MLR, and concluded that the suppressor cell was of T cell origin. Rodriguez et al. (3) used similar negative selection techniques and treated neonatal spleen cells with a variety of antibodies and complement. The latter investigators characterized the neonatal cell capable of suppressing the MLR as a null cell (neither T nor B cell). Furthermore, these authors found that purified neonatal T cells possessed normal reactivity in the MLR, and that neonatal spleen cells from nude mice could also suppress the MLR (3). Peeler et al. (4) used velocity sedimentation to isolate suppressor cells from the neonatal spleen that were histologically characterized as macrophages and mast cells. Oseroff et al. (5) used the panning technique in an attempt to characterize the neonatal suppressor cell. These

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[1] *Abbreviations used in this paper:* CAS, concanavalin A–stimulated rat spleen cell supernatant; EA, antibody-coated erythrocyte; EAC, antibody and complement–coated erythrocyte; FACS, fluorescence-activated cell sorter; FcR, Fc receptor; FCS, fetal calf serum; FITC-GAR, fluorescein isothiocyanate–conjugated goat anti–rat Ig; GVHD, graft-vs.-host disease; Il-2, interleukin 2; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; MLR, mixed leukocyte reaction; NK, natural killer; NS, natural suppressor; PEC, peritoneal exudate cells; PHA, phytohemagglutinin; PSG, penicillin, streptomycin, and glutamine–containing medium; SRBC, sheep red blood cell; TLI, total lymphoid irradiation.

authors found that neonatal cells positively selected with antibodies against the Thy-1.2, Ig, MAC-1, or F4/80 surface markers did not suppress an MLR, but that negatively selected cells suppressed as well as unfractionated cells. The results indicated that the neonatal suppressor cells resided in the null cell population.

We report here the development of long-term cloned cell lines derived from the neonatal spleen that maintain suppressive activity in the MLR. The experimental data suggest that these cloned natural suppressor (NS) cells have a unique regulatory function and surface phenotype.

Materials and Methods

Animals. BALB/c (H-2^d), C57BL/Ka (H-2^b), and Swiss/nude mice were obtained from the specific pathogen–free colony of Robert Kallman, Department of Radiology, Stanford University, Palo Alto, CA. BALB/c neonates, 1–3 d old, were obtained from the same source. A/J (H-2^a) mice were a kind gift of C. G. Fathman, Department of Medicine, Stanford University. DBA/2 (H-2^d) mice were purchased from Simonsen Laboratories, Inc. (Gilroy, CA). C3H/HeJ (H-2^k) mice, 5–8 wk old, were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice used in experiments were housed in conventional animal facilities and were 6–10 wk of age. Male Lewis rats, 8–10 wk old, were purchased from Simonsen Laboratories, Inc.

Preparation of Spleen Cells. Single-cell suspensions of spleen cells were prepared using a sterile technique. Upon removal, spleens were gently pressed through nylon fiber mesh (Tetko, Inc., Elmsford, NY) then centrifuged and resuspended in RPMI-1640 (Gibco, Grand Island, NY). Cells were counted and viability determined by trypan blue dye exclusion.

MLR Suppressor Assay. Irradiated (3,000 rad) cloned cell lines (control or NS cells) were added at a variety of concentrations to 96-well, flat-bottom microtiter plates (Costar, Cambridge, MA) containing 5×10^5 responder spleen cells and 5×10^5 irradiated (3,000 rad) stimulator spleen cells/well in a final volume of 300 μl. Stimulator and cocultured cells were irradiated in a ^{137}Cs irradiator (Mark 1 Model 25 Irradiator; J. L. Shepherd and Associates, Glendale, CA). MLR media consisted of RPMI-1640, 10% human serum (Biocell Laboratories, Carson City, CA), 5×10^{-5} M 2-mercaptoethanol (2-ME), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (PSG), all from Gibco. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 120 h, plates were pulsed with 1 μCi/well [^{3}H]thymidine (sp act 6.7 Ci/mM; New England Nuclear, Boston, MA). After 18 h, the plates were harvested onto glass fiber filter papers (Whatman, Inc., Clifton, NJ) by means of a multiple automated sample harvester (Bio-Plastics, Redwood City, CA). The dried paper disks were placed into vials containing 2 ml scintillation cocktail (Betafluor; National Diagnostics, Somerville, NJ) and incorporated radioactvity was measured in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). The data was expressed as the mean of triplicate cultures. Percent suppression was calculated as: [1 − (cpm with cocultured cells)/(cpm without cocultured cells)] × 100.

Preparation of Concanavalin A–stimulated Rat Spleen Cell Supernatants (CAS). Lectin-free supernatants from concanavalin A–stimulated Lewis rat spleen cells were prepared as previously described by Oseroff et al. (5).

Cell Lines. The HT-2 cell line (17), an interleukin 2 (IL-2)-dependent T cell line of BALB/c origin was obtained from C. G. Fathman, Department of Medicine, Stanford University, and maintained in RPMI-1640 media containing 15% CAS, 10% fetal calf serum (FCS) (Hyclone, Logan, UT), 2-ME, and PSG. The 4.1.12 T cell clone, which is an A/J anti-C57BL/Ka–reactive helper cell line was obtained from C. G. Fathman. These cells were periodically divided and propagated by adding 10^6 T cells to 5.0×10^7 irradiated C57BL/Ka spleen cells in RPMI-1640 with 10% FCS, 2-ME, PSG, and 1% CAS. The 5.2 cloned T cell line, a BALB/c line reactive against ovalbumin, was obtained from C.

Janeway, Department of Pathology, Yale University. The 5.2 cell line was periodically propagated by adding 10^6 T cells to 2.0×10^7 irradiated BALB/c spleen cells, with ovalbumin, in Click's medium plus 10% CAS. The YAC-1 and EL4 tumor cell lines were obtained from I. L. Weissman, Department of Pathology, Stanford University, and maintained in RPMI-1640 with 10% FCS, 2-ME, and PSG.

Antibodies. Monoclonal rat anti-mouse antibodies specific for Lyt-1 (clone 53-7.3) and Lyt-2 (53-6.7) were obtained from Becton-Dickinson, Inc. (Sunnyvale, CA). Monoclonal rat anti-mouse antibody against Ly-5 (M 1/9.3HL) was purchased from Hybritech (San Diego, CA). Ly-5 (CLA-T200) is found on all leukocytes (18). Monoclonal rat anti-mouse antibody against MAC-1 was provided by J. Monaco, Department of Medical Microbiology, Stanford University. Monoclonal rat anti-mouse antibody against Thy-1.2 (clone 30-H12) was obtained from I. L. Weissman, Department of Pathology, Stanford University. Monoclonal rat anti-mouse idiotypic antibody, used as an irrelevant control, was obtained from R. Levy, Department of Medicine, Stanford University. Fluoresceinated goat anti–rat Ig (FITC-GAR), which was absorbed to have no crossreactivity with mouse Ig, was purchased from Capell Laboratories (Cochranville, PA). FITC-GAR was used as a second antibody for previously mentioned rat anti-mouse monoclonal antibodies, which were not fluoresceinated. Rabbit anti–mouse Ig antiserum was prepared as described previously (19). Rabbit anti–asialo GM1 antiserum was purchased from Wako Chemicals USA, Inc. (Dallas, TX). Fluoresceinated goat anti–rabbit Ig, used as a second stage for the above-mentioned rabbit antibodies, was purchased from Capell Laboratories. Specifically eluted rabbit anti–keyhole limpet hemocyanin antibodies were used as control antibodies for staining with rabbit-derived antibodies (19). Monoclonal mouse anti-IE^d (clone 14.4.4) antibody was obtained from H. O. McDevitt, Department of Medical Microbiology, Stanford University, and conjugated to fluorescein by a standard protocol (20). Directly fluoresceinated mouse anti-human Leu-3 antibody (Becton-Dickinson) was used as a control for staining with fluoresceinated mouse anti-IE^d.

Immunofluorescent Staining and Fluorescence-activated Cell Sorter (FACS) Analysis. Spleen cells were treated to lyse red cells (0.155 M NH_4Cl, 0.1 M $KHCO_3$, and 0.1 mM EDTA in distilled water, pH 7.4), or purified by density gradient centrifugation on Lympholyte-M (Cederlane Labs, Ontario, Canada) before use for staining. The entire staining procedure was performed at 4°C. Indirect staining was performed using a modification of a two-stage procedure described previously (5). All antibodies were used at a concentration previously shown to be at the saturation point for staining. Staining with directly fluorescinated antibodies was performed in a similar manner, except the second stage was not used.

The stained cells were analyzed on a FACS III (Becton Dickinson) that was coupled to a PDP-11 computer. Scatter gates were set to exclude residual erythrocytes, dead cells, and aggregates. Light scatter and autofluorescence were determined on unstained but identically manipulated cells. Staining controls included cells stained with irrelevant first-stage antibodies. In general, integration of antibody staining curves to determine percent positivity were performed at the point where background staining with irrelevant antibody was ~2%.

Mycoplasma Testing. Cell lines were examined for the presence of mycoplasma using the technique described by Chen (21). In essence, supernatants from cell lines to be tested for mycoplasma were first incubated with the 3T3 (22) mycoplasma-free indicator cell line. The indicator cells were then stained with Hoechst 33258 reagent, which detects the presence of mycoplasma DNA on the surface of indicator cells incubated with test supernatants. All cell lines used in this paper were mycoplasma negative at all times tested.

Natural Killer (NK) Assay. The NK assay against the YAC-1 tumor cell line was performed as previously described (5). In this assay, 10^4 ^{51}Cr-labeled YAC-1 cells were incubated for 4–6 h with effector cells at a variety of different effector/target cell ratios. Supernatants were harvested and counted on a gamma counter (Biogamma II, Beckman Instruments, Inc.). Percent specific lysis was calculated according to the formula: percent specific lysis = [(cpm experimental) − (cpm spontaneous release)] × 100/[(cpm maximum

release) − (cpm spontaneous release)]. Swiss nude mouse spleen cells were used as a positive control.

Proliferation of HT-2 Cells in Response to IL-2. The HT-2 cell line can be used to measure the presence of IL-2 in test supernatants (23). For this purpose, 2×10^4 HT-2 cells/well were plated into 96-well microtiter plates in media consisting of RPMI-1640, 5×10^{-5} M 2-ME, 10% FCS, and PSG. Various dilutions of a control IL-2-containing supernatant were added, as were similar dilutions of supernatants to be tested for the presence of IL-2. After 20 h, the cultures were pulsed with [^{3}H]thymidine, then harvested six h later and counted as described for the MLR. Assays for suppression of [^{3}H]thymidine uptake by various irradiated cocultured cells was performed using the same procedure, except that a constant source of IL-2 (20% CAS) was added, and various numbers of irradiated cocultured cells were added per well.

Fc and C3 Receptor Assay. The assays for detecting Fc and C3 receptors on the surface of NS and control cells were performed as previously described (24).

Thymocyte Proliferation Assay. The thymocyte proliferation assay was performed according to the protocol described by Mizel (25). Thymus cells of C3H/HeJ mice (5–8 wk old) were plated into microtiter plates at 1.5×10^6 cells/well in media consisting of RPMI-1640, 10% FCS, 2-ME, PSG, and phytohemagglutinin (PHA) (1.25 μg/ml) (Wellcome Research Laboratories, Beckenham, Great Britain). In assays measuring the potency of IL-1 preparations, the supernatants to be tested were added at various dilutions. After 3 d, cultures were pulsed with 0.5 μCi/well [^{3}H]thymidine (as used in the MLR) and harvested 6 h later.

IL-1 Production Suppressor Assay. Peritoneal exudate cells (PEC) or resident peritoneal macrophages from BALB/c mice were tested for the ability to produce IL-1 in the presence of NS cells, control cells, or in the absence of added coculture cells. PEC or resident macrophages were plated into 24-well plates (Costar) at a density of 10^6 cells/well in media consisting of RPMI-1640, 10% FCS, and PSG. After 2 h, the nonadherent cells were removed by vigorous washing. The adherent cells were then incubated in "induction" media, which consisted of RPMI-1640, 5% FCS, PSG, lipopolysaccharide (LPS) (10 μg/ml) (Difco, Detroit, MI) and indomethacin (7 μg/ml) (Sigma Chemical Co., St. Louis, MO). At this point, graded numbers of irradiated NS cells, control cells, or no cells were added to the cultures. After 24 h, the supernatants were removed, dialyzed, sterilized, and tested for IL-1 activity in the thymocyte proliferation assay (as described). Supernatants were tested in the HT-2 assay to confirm the absence of IL-2 activity.

Establishment of Neonatally Derived NS Cell Lines. Neonatally derived NS cell lines were established using a modification of the procedure described by Oseroff et al. (5). Spleen cells were obtained from BALB/c pups <3 d old, and prepared as single cell suspension in culture media consisting of RPMI-1640, 10% FCS, 5×10^{-5} M 2-ME, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml concanavalin A (Pharmacia Fine Chemicals, Piscataway, NJ). Neonatal cells (5×10^6 cells/well) were added to 24-well plates (Costar) in 1 ml/well. After 3–6 d, this medium was changed by gentle aspiration with a transfer pipet. Cells were then fed with RPMI-1640, 10% FCS, 2-ME, PSG, and 30% CAS. Medium was changed daily by gentle aspiration and replacement with fresh medium. Irradiated feeder cells (BALB/c adult spleen; 3,000 rad) were added on a weekly basis, until healthy proliferating cultures were established. After the first 6 wk, addition of feeder cells was no longer necessary. Initially, several different types of adherent and nonadherent cells were seen in culture, similar to those reported by Oseroff et al. (5) in the establishment of NS cell lines derived from mice treated with TLI. After ~6–8 wk in culture, a predominant cell type arose. These cells were of medium size, and were mildly adherent to plastic. At this point, the predominant proliferating cells were transferred from 24-well plates to 25 cm^2 flasks (Corning Glass Works, Corning, NY). Cells established with this procedure have been in culture for >1 yr. These cells were highly suppressive when added to the MLR at all time points tested (see Results). The cells continue to require daily feeding with fresh media containing 10–15% CAS, and die within several days after removal of CAS from the tissue culture medium.

Initially, four uncloned, neonatally derived NS cell lines were established using this

protocol. After 5 mo, one uncloned cell line (N4B) was cloned by limiting dilutions in 96-well microtiter plates (0.3 cells/well) using a protocol described by Hertel-Wulff et al. (26). All cell lines were tested for mycoplasma contamination using the method of Chen (21) and found to be free of detectable levels of mycoplasma.

Results

Suppression of MLR. Aliquots of fresh neonatal spleen cells from BALB/c pups <3 d old were tested for suppressive activity in the MLR. In a representative experiment (Fig. 1 *A*), addition of irradiated (3,000 rad in vitro) neonatal spleen cells, at a 1:1 ratio to adult responder cells, resulted in 75% suppression of the MLR compared to that observed without cocultured cells. Addition of irradiated adult spleen cells produced minimal suppression as compared to the neonatal cells (Fig. 1 *A*).

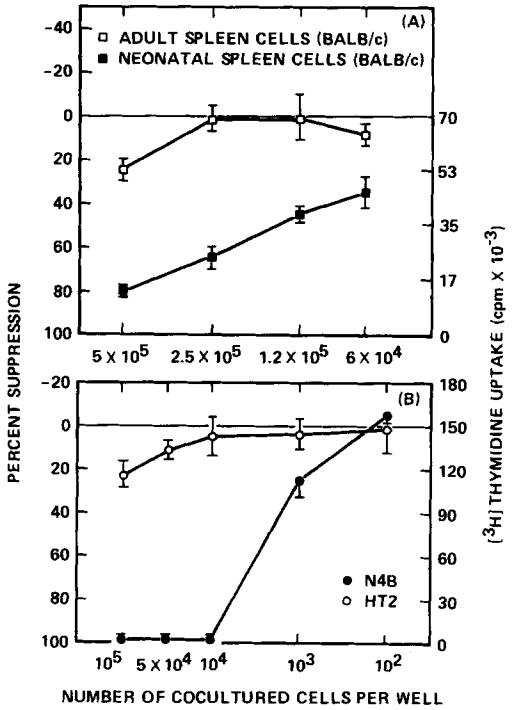

FIGURE 1. (*A*) Suppression of the MLR after addition of fresh BALB/c neonatal (<3 d old) spleen cells. The BALB/c vs. C57BL/Ka MLR assay was performed and results evaluated as described for *B*. Graded numbers of irradiated BALB/c neonatal spleen cells (■—■) or irradiated adult (2 mo old) BALB/c spleen cells (□—□) were added to the MLR. BALB/c vs. C57BL/Ka cultures without cocultured cells gave 69,200 ± 978 cpm. BALB/c vs. BALB/c cultures gave 3,066± 643 cpm. A representative experiment is depicted. (*B*) Suppression of the MLR after the addition of uncloned natural suppressor (NS) cells derived from the spleen of BALB/c neonatal mice. Graded numbers of irradiated (3,000 rad) NS cells or irradiated HT-2 cells were added to a BALB/c vs. C57BL/Ka MLR with 5×10^5 responder cells and 5×10^5 irradiated (3,000 rad) stimulator cells/well. Cultures were pulsed with [^{3}H]thymidine on day 5, and harvested 18 h later. (●—●), N4B (uncloned NS cell line): (○—○), HT-2 (a cloned BALB/c T cell line). Percent suppression is calculated as [1 − (cpm with cocultured cells)/(cpm without cocultured cells)] × 100. BALB/c vs. C57BL/Ka cultures without cocultured cells gave 149,000 ± 8,997 cpm. BALB/c vs. BALB/c cultures gave 2,680 ± 320 cpm. Results are given as the mean ± SE of triplicate cultures. A representative experiment is shown.

Initially, four uncloned natural suppressor (NS) cell lines were established from four different groups of neonatal BALB/c pups, as described in Materials and Methods. There was an initial paucity of cells until the emergence of rapid cell growth at ~2 mo after the initiation of cell culture. Thus, 2 mo was the earliest time at which suppressive activity was tested in the MLR. Fig. 1 *B* shows an experiment in which the N4B uncloned cell line (irradiated in vitro with 3,000 rad) was added to a BALB/c vs. C57BL/Ka MLR after in vitro culture for 2–4 mo. Even at a suppressor/responder ratio of 1:50, almost 100% suppression of the MLR is seen. The BALB/c cloned T cell line, HT-2, was used as a cocultured cell control in this experiment, since these cells, as well as the NS cells are dependent upon the addition of CAS to the culture medium for continued growth. Although 23% suppression was observed with 10^5 HT-2 cells/well, minimal suppression was observed at lower cell doses. The N4B line was highly suppressive at these lower doses (Fig. 1 *B*).

After the N4B cell line had been in culture for ~5 mo, cloning was performed using limiting-dilution techniques (see Materials and Methods). Six cloned long-term cell lines were derived from the N4B cell line, and all suppressed the MLR. Each of the two cloned NS cell lines showing greatest suppression upon initial examination in the MLR were further examined in 12 separate MLR assays. Fig. 2 is representative of eight experiments in which the 4BA4 cloned NS cell line was added to syngeneic responder cells (BALB/c) in the MLR. Marked suppression (98%) of the MLR is seen when 10^4 4BA4 cells were added. The suppression of the MLR starts to taper off when $<10^4$ cells/well are added. The HT-2 control cell line (10^4 cells/well) exhibited no suppression, and actually enhanced [^{3}H]-thymidine uptake in this particular assay. The 4BA4 and 4BC9 cloned NS cell lines were tested in four separate MLR assays in which the NS cells were allogeneic to the responder cell population (C5BL/Ka vs. DBA/2 or C57BL/Ka vs. BALB/c). In one representative experiment, 10^4 4BA4 and 4BC9, cloned NS

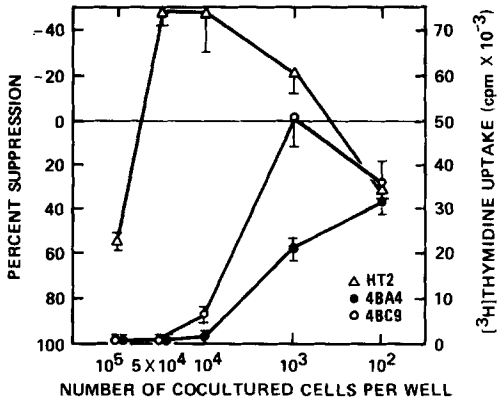

FIGURE 2. Suppression of the BALB/c vs. C57BL/Ka MLR after the addition of neonatally derived cloned BALB/c NS cell lines. The MLR was performed and results evaluated as described in Fig. 1. Graded numbers of irradiated 4BA4 (●—●), a neonatally derived cloned NS cell line; 4BC9 (○—○), a neonatally derived cloned NS cell line; or HT-2 (△—△), a BALB/c cloned T cell line cells were added to the MLR. BALB/c vs. C57BL/Ka cultures without cocultured cells gave 48,357 ± 4,797 cpm. BALB/c vs. BALB/c cultures resulted in 3,607 ± 370 cpm. A representative experiment is depicted.

cells showed a mean of 96% and 82% suppression, respectively. In this experiment, enhancement of the MLR response was seen when 10^4 HT-2 cells were added. These results demonstrate that the cloned suppressor cell lines suppress the MLR regardless of whether the H-2 haplotype is matched or mismatched with the responder or stimulator cell population. In all 12 MLR assays, the addition of 10^4 4BA4 cells/well gave a mean suppression of 94%, as compared to 3% suppression with the addition of 10^4 control cultured cells (HT-2 cells, or the A/J T cell clone, 4.1.12).

HT-2 Proliferation in the Presence of NS Cells. Possible mechanisms whereby the cloned neonatal NS cell lines could be suppressing the MLR include nonspecific inhibition of the uptake of [^{3}H]thymidine, or absorption of IL-2 from the culture medium. To examine these possibilities, various doses of irradiated 4BA4 or 4BC9 cell lines were added to a fixed number of HT-2 cells in the presence of a source of IL-2 (CAS). The HT-2 cell line is derived from a helper T cell line of BALB/c origin which proliferates in response to IL-2 (17). As shown in Fig. 3, there is only a small difference in suppression of the [^{3}H]thymidine uptake of HT-2 cells seen when the 4BA4 or 4BC9 cell lines are added, in comparison to the suppression seen in the presence of irradiated control cocultured cells (BALB/c T helper cells reactive against ovalbumin, the 5.2 cell line). To determine whether cloned NS cells can inhibit proliferation of HT-2 cells when IL-2 is present in suboptimal quantities, 10^4 irradiated 4BA4 cells were added to a fixed number of HT-2 cells in the presence of various dilutions of CAS. In three separate assays, a full dose-response curve was plotted using the [^{3}H]thymidine uptake of HT-2 cells incubated in serial twofold dilutions of CAS, with and without 4BA4 cells. Addition of the 4BA4 cells altered the curve minimally (shift of less than one twofold dilution) in all assays (data not shown). However, >90%

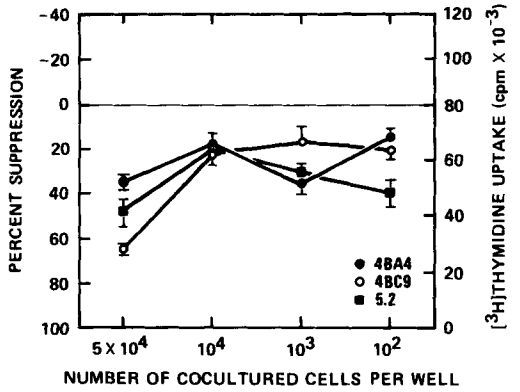

FIGURE 3. The effect of cloned NS cell lines on [^{3}H]thymidine incorporation by proliferating HT-2 cells. Graded numbers of irradiated 4BA4 (●—●), a neonatally derived cloned BALB/c NS cell line; 4BC9 (○—○), a neonatally derived cloned BALB/c NS cell line; or 5.2 (■—■), a BALB/c T helper cell line cells were added to HT-2 cells (2×10^4 cells/well) in the presence of 15% CAS. Cultures were pulsed with [^{3}H]thymidine 20 h after the initiation of the assay, and harvested 6 h later. Percent suppression was calculated by comparing [^{3}H]thymidine uptake without cocultured cells to that seen with cocultured cells. HT-2 cultures in the presence of IL-2 gave 81,874 ± 3,354 cpm. A representative experiment is shown.

suppression of the MLR was observed with the addition of similar numbers of cloned NS cells (Fig. 2).

We also examined the ability of irradiated HT-2 vs. irradiated 4BA4 NS cells to absorb IL-2 from the culture media. Irradiated HT-2 or 4BA4 cells were plated into 96-well microtiter plates at a density of 10^5 or 10^4 cells/well in media containing 20% CAS. After 24 h, the supernatants were removed and tested for IL-2 activity in the HT-2 assay, as shown in Fig. 4. While 10^5 irradiated HT-2 or 4BA4 absorbed substantial quantities of IL-2 from the culture media, 10^4 irradiated HT-2 or 4BA4 did not absorb sufficient IL-2 from the culture media to alter the dilution curve compared to unabsorbed control CAS-containing media. Thus, at cell numbers where NS cells suppress the MLR by almost 100%, and HT-2 cells show little or no suppression of the MLR (10^4 cells/well), both of these cell lines absorbed little or no IL-2 from the culture media. These experiments demonstrate that nonspecific inhibition of [^{3}H]thymidine uptake or absorption of the IL-2 does not explain the suppression of the MLR by cloned NS cells.

Effect of NS Cells on IL-1 Production. Graded numbers of irradiated NS cells or HT-2 cells were added to resident peritoneal macrophages or peritoneal exudate macrophages to assess the effect on IL-1 production. Supernatants were collected from PEC or resident macrophages that were induced to produce IL-1 (as described in Materials and Methods) in the presence or absence of cocultured cells, and the supernatants were tested in the thymocyte proliferation assay to detect the presence of IL-1. All supernatants were also tested in the HT-2 assay, and had no detectable IL-2 activity. The data presented in Table I demonstrates that the addition of 4BA4 NS cells to resident peritoneal macrophages does not interfere with the ability of these cells to produce IL-1 after stimulation with LPS. Essentially identical results were obtained with PEC (data not shown). Table I also shows that NS cells do not produce IL-1 under conditions in which macrophages are induced to produce IL-1.

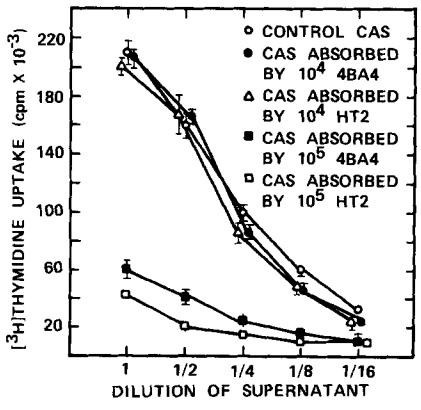

FIGURE 4. The ability of NS cells and HT-2 cells to absorb IL-2. Irradiated 4BA4 NS cells or HT-2 cells were plated into microtiter wells at a density of 10^4 or 10^5 cells/well in media containing 20% CAS. 24 h later, the supernatants were harvested and tested for the presence of IL-2 in the HT-2 assay. (○—○), unabsorbed CAS; (●—●), CAS absorbed by 10^4 4BA4 cells; (△—△), CAS absorbed by 10^4 HT-2 cells; (■—■), CAS absorbed by 10^5 4BA4 cells; (□—□), CAS absorbed by 10^5 HT-2 cells.

TABLE I
*Effect of NS Cells on IL-1 Production by Resident Peritoneal Macrophages (RPM)**

Culture conditions	[^{3}H]Thymidine uptake‡
RPM plus LPS	8,921 ± 602
RPM alone	650 ± 135
RPM plus LPS and 10^5 4BA4	13,339 ± 1,023
RPM plus LPS and 10^4 4BA4	10,320 ± 174
RPM plus LPS and 10^3 4BA4	9,037 ± 615
RPM plus LPS and 10^5 HT-2	10,529 ± 659
RPM plus LPS and 10^4 HT-2	9,447 ± 510
RPM plus LPS and 10^3 HT-2	11,393 ± 888
10^5 4BA4 plus LPS	766 ± 65
10^5 4BA4	413 ± 41
10^5 HT-2 plus LPS	943 ± 122
10^5 HT-2	441 ± 54
RPM plus 10^5 4BA4	660 ± 116
Medium plus LPS	845 ± 22

* RPM, resident peritoneal macrophage.
‡ [^{3}H]Thymidine uptake by C3H/HeJ thymocytes incubated in a 10% solution of the given supernatant, in the presence of PHA; cpm ± SE.

Surface Phenotype of Cloned NS Cells. The cell surface phenotype of the 4BA4 cloned cell line was determined after staining with a panel of antibodies and analysis on the FACS III. Table II shows representative staining results of several different experiments. The cells stained brightly with anti-Thy-1.2, anti-Ly-5, and anti–asialo GM1 antibodies. However, the staining was essentially negative for surface Ig and I-E, Lyt-1, Lyt-2, and MAC-1 surface antigens. The uncloned parental cell line N4B (from which 4BA4 was cloned) showed an identical staining profile when first assayed, ~2 mo after the initiation of culture.

The 4BA4 and 4BC9 cell lines were tested for the presence of the Ig Fc fragment receptor (FcR) and the complement (C3) receptor. Rosette assays were performed using sheep red blood cells (SRBC) coated with hyperimmune mouse anti SRBC antiserum (IgG-EA), or SRBC coated with rabbit IgM anti-SRBC plus AKR mouse serum, which included complement (IgM-EAC). The 4BA4 and 4BC9 cell lines did not form rosettes with SRBC, IgM-EA, IgM-EAC, or IgG-EA. Thus, neither line expressed Fc or C3 receptors. The P388D1 macrophage tumor cell line (27) was used as a positive control. The latter cells formed 92% rosettes with IgG-EA and 64% with IgM-EAC in a representative experiment.

Assay for NK Activity. In view of the similarities between the staining pattern of the cloned neonatal suppressor cell lines with that reported for cloned murine NK cells (28), the 4BA4 cloned NS line was tested for NK activity against the NK-sensitive YAC-1 tumor cell line. Several laboratories with established long-term NK cell lines report vigorous lysis of the YAC-1 cell line by cloned NK cell lines (28–30). Fig. 5 shows the results representative of five experiments, in

TABLE II

Surface Phenotype of the Neonatally Derived Cloned 4BA4 NS Cell Line

Antibodies used for staining	Positively stained cells*	
	4BA4	BALB/c adult spleen cells
	%	
Anti-Thy-1.2	88 ± 6	43 ± 9
Anti-Lyt-1	1 ± 1	26 ± 2
Anti-Lyt-2	2 ± 2	15 ± 3
Anti-Ig	4 ± 3	40 ± 10
Anti-IE	4 ± 1	ND‡
Anti-MAC-1	1 ± 1	ND§
Anti-Ly-5	88 ± 7	87 ± 3
Anti-asialo-GM1	72 ± 11	ND‖

* Mean ± SE positive cells above background fluorescence for 3–6 separate experiments.

‡ ND, not done. BCL_{5b}, a B cell leukemia cell line, stained 84 ± 10% positive.

§ P388D1, a macrophage tumor cell line, stained 72 ± 11% positive.

‖ Swiss nude spleen cells stained 45 ± 2% positive.

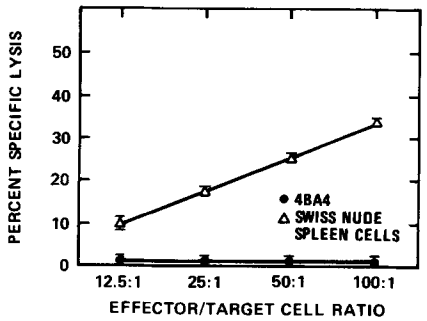

FIGURE 5. Percent specific lysis of ^{51}Cr-labeled YAC-1 cells (10^4 cells/well) after a 4-h incubation with different numbers of effector cells. (●—●), 4BA4 neonatally derived NS cell line; (△—△), Swiss nude spleen cells.

which the 4BA4 cell line showed no lysis of YAC-1 target cells above background. Similar results were obtained when the uncloned parental N4B cell line was tested for NK activity.

Discussion

In recent years, investigators have demonstrated (1–12) the existence of nonspecific suppressor cells in the neonatal mouse spleen that inhibit T cell alloreactivity and a variety of other immune functions. In this paper, we describe the establishment and characterization of cloned NS cell lines derived from the spleen of neonatal BALB/c mice. Irradiated cloned NS cells suppress the MLR between normal adult responder and stimulator cells with a 50-fold greater

efficacy than irradiated fresh neonatal spleen cells. Both fresh neonatal spleen cells and cloned NS cells suppress the MLR regardless of the H-2 haplotype of the responder or stimulator cell. Cloned NS cells show 80–100% suppression of the MLR over a range of cell doses in which control T cell lines derived from BALB/c and other strains show little or no suppression.

Continued growth of the cloned NS cells is dependent upon the addition of supernatants of concanavalin A–stimulated rat spleen cells (CAS) to the culture medium. NS cells die within several days after the removal of CAS. Preliminary experiments indicate that substitution of recombinant IL-3 (kindly supplied by DNAX Research Institute, Palo Alto, CA) for CAS is not sufficient to support growth of cloned NS cells. However, the substitution of partially purified human IL-2 (Collaborative Research, Lexington, MA) does support the growth of cloned NS cells (R. Schwadron, unpublished observations).

We routinely tested all cell lines described in this paper for the presence of mycoplasma contamination (as described in Materials and Methods), and all cell lines were found to be mycoplasma-free. In addition, cloned NS cells were also tested by the University of California, San Francisco cell culture facility and found to be mycoplasma-free in three independent tests. Thus, negative serial assays for mycoplasma indicate that the cloned NS cells and their uncloned parental cells never contained detectable mycoplasma during any time from their initial inception to the present.

Cloned NS cells did not significantly inhibit [^{3}H]thymidine uptake by proliferating HT-2 cells, compared to control cocultured cells. This suggests that interference with [^{3}H]thymidine uptake per se can not explain the suppression of the MLR. Furthermore, experiments in which cloned NS cells were added to HT-2 cells in various dilutions of CAS indicate that cloned NS cells do not inhibit proliferation of activated T cells in response to IL-2. The latter experiments also indicate that doses of NS cells (10^4) that inhibit the MLR do not absorb or consume sufficient IL-2 to change the dilution curve in the HT-2 assay. Experiments examining the ability of irradiated NS cells and irradiated HT-2 cells to consume IL-2 from culture media during a 24-h interval demonstrate little or no difference in IL-2 consumption. Thus, IL-2 absorption or consumption cannot explain the ability of NS cells to suppress the MLR when similar numbers of HT-2 cells cannot.

The addition of cloned NS cells does not interfere with the ability of peritoneal macrophages (thioglycolate-stimulated or resident) to produce IL-1. However, the addition of cloned NS cells results in vigorous suppression of the MLR. Since the number of cells used ($\leq 10^4$) does not prevent IL-1 secretion by macrophages, nor interferes with T cell (HT-2 cell) proliferation in response to IL-2, it is tempting to speculate that suppression of the MLR is related to interference with early stages of T cell activation. In particular, NS cells may suppress secretion of IL-2 and/or the induction of IL-2 receptors during T cell activation.

Immunofluorescence staining and FACS analysis revealed that cloned NS cells were strongly positive for the cell surface antigens Thy-1.2, Ly-5 (CLA T200), and asialo-GM1. NS cells did not express detectable surface Ig, MAC-1, IE, Lyt-1, or Lyt-2 surface antigens, nor Fc or C3 receptors. Although the NS cells are strongly positive for Thy-1.2, the absence of Lyt-1 and Lyt-2 antigens on the

cloned NS cell lines and the parental line indicates that these cells are not mature T cells. The absence of surface Ig, MAC-1 antigen, and Fc or C3 receptors, and the presence of Thy-1.2 antigen argues against a macrophage, monocyte, or B cell origin for cloned NS cells.

The surface phenotype of the cloned NS cells is similar to that reported previously for cloned NK cell lines (28, 31). However, NS cells did not lyse ^{51}Cr-labeled YAC-1 target cells. In contrast, vigorous lysis of these target cells has been reported for cloned NK cell lines (28–30).

The in vivo function of the cloned NS cells and their relationship to the suppressor cells found in the fresh neonatal spleen await further investigation. We have preliminary evidence that cloned NS cells can suppress in vivo GVHD in neonates, as measured by the spleen enlargement assay of Simonsen (32) (work in progress). The relationship to the fresh neonatal suppressor cell(s) is complicated by the controversy surrounding the nature of these cells. Various authors have concluded that the neonatal suppressor cells are T cells (1), macrophages/monocytes (4), mast cells (4), or null cells (3, 5). An examination of these reports suggests that several different types of suppressor cells may exist in the neonatal spleen, with different suppressor/effector functions. It appears likely that suppressor cells in the neonate play a role in the development of tolerance to foreign antigens (33), and may also be involved in the maintainance of immunologic balance between the mother and F_1 fetal graft (34). The development of cloned suppressor cell lines may help elucidate the regulatory functions that contribute to the heightened susceptibility of tolerance induction in the neonate.

Summary

The establishment and characterization of cloned natural suppressor (NS) cell lines derived from the spleen of neonatal BALB/c mice are described. Cloned NS cells suppress the mixed leukocyte reaction (MLR) between normal adult responder and stimulator spleen cells with a 50-fold greater efficiency than fresh neonatal cells. Suppressive activity of both cells did not depend on the haplotype of the responder or stimulator cells, and was radioresistant. Cloned NS cells did not inhibit the uptake of [^{3}H]thymidine by HT-2 cells proliferating in response to interleukin 2 (IL-2), nor the in vitro secretion of IL-1 by macrophages in response to lipopolysaccharide. Several experiments indicated that absorption of IL-2 could not explain the suppression of the MLR by the NS cells in the range of cell numbers tested. The results suggest that NS cells may suppress the MLR by interfering with early stages of T cell activation. The cell surface of a cloned NS cell line was examined using immunofluorescence staining, and was strongly positive for the Thy-1.2, Ly-5, and asialo-GM1 antigens. However, Lyt-1, Lyt-2, surface Ig, IE, MAC-1, and Fc and C3 receptor markers were not detected. In addition, NS cells showed no cytolytic activity against the YAC-1 target cell line. On the basis of these findings, cloned NS cells do not appear to be mature T cells, B cells, macrophages, or NK cells. The development of cloned NS cells may be useful in determining the identity and mechanism of action of nonspecific suppressor cells in the neonatal spleen, and their role in neonatal tolerance and maternal-fetal relationships.

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