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Nerve Growth Factor Is an Autocrine Survival Factor for Memory B Lymphocytes

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Summary

Production of nerve growth factor (NGF) was assessed in cultures of human T and B lymphocytes and macrophages. NGF was constitutively produced by B cells only, which also expressed surface p140$^{trk\text{-}A}$ and p75^{NGFR} molecules and hence efficiently bound and internalized the cytokine. Neutralization of endogenous NGF caused disappearance of Bcl-2 protein and apoptotic death of resting lymphocytes bearing surface IgG or IgA, a population comprising memory cells, while surface IgM/IgD "virgin" B lymphocytes were not affected. In vivo administration of neutralizing anti-NGF antibodies caused strong reduction in the titer of specific IgG in mice immunized with tetanus toxoid, nitrophenol, or arsonate and reduced numbers of surface IgG or IgA B lymphocytes. Thus, NGF is an autocrine survival factor for memory B lymphocytes.

Introduction

Nerve growth factor (NGF), described and characterized more than 30 years ago (Levi-Montalcini, 1987; Cohen, 1960) as the first soluble signal mediating intercellular communications, is a member of the family of proteins known as neurotrophins, which are critical for regulated development and survival of neuronal cells (Barde, 1990). Its role in maintaining survival of neurons from sympathetic and sensory ganglia has been established for decades (Levi-Montalcini and Angeletti, 1966, 1968). In recent years, these initial observations have been extended to other populations of central nervous system neurons, including basal forebrain cholinergic cells (Johnson et al., 1986; Shelton and Reichardt, 1986). It is believed that NGF, like the other neurotrophins, is produced in the nervous system by accessory cells, such as glial cells and oligodendrocytes (Ernfors et al., 1990; Hofer et al., 1990), which therefore regulate the differentiation process of neurons mostly through the formation of paracrine circuits.

The neurotrophins, including NGF, brain-derived neurotrophic factor, neurotrophin-3 (NT-3), and NT-4/5, exert their effects on cellular targets via specific surface receptors, which, upon binding and internalization of the ligand, trigger a cascade of biochemical events, representing the adaptive response (reviewed by Barde, 1990). Two classes of neurotrophin-binding sites can be identified on target cells, based on low ($K_d \approx 1$ nM) and high ($K_d \approx 20$ pM) affinity for the ligand or ligands. The molecular nature of these receptors has been recently characterized (Meakin and Shooter, 1992). A 75 kDa glycoprotein, named p75^{NGFR}, mediates low affinity binding of any neurotrophin with similar affinity (Chao, 1994). Proteins belonging to the family of trk tyrosine kinase receptors, which interact with neurotrophins in a highly specific manner, are responsible for high affinity binding. p140$^{trk\text{-}A}$ combines with NGF, p140$^{trk\text{-}B}$ with brain-derived neurotrophic factor, and p140$^{trk\text{-}C}$ with NT-3 and NT-4/5 (Barbacid, 1994). Probably, each class of receptors conveys distinct signals to the target cell, since it appears that p75^{NGFR} and p140$^{trk\text{-}A}$ do not form NGF-binding heterodimers (Jing et al., 1992). Interestingly, p75^{NGFR} displays structural homology to the tumor necrosis factor receptors 1 and 2, the lymphocyte surface antigens CD30, CD40, OX40, and Fas/Apo-1 surface antigen, molecules involved in preventing or mediating apoptosis (reviewed by Raffioni et al., 1993).

Since the first description of the purification of NGF from mouse maxillary gland cells, it was evident that this factor was also produced by several nonnervous cell types (Levi-Montalcini, 1987), including keratinocytes (Di Marco et al., 1993) and smooth muscle cells (Ueyama et al., 1993). Likewise, expression of the *trk* proto-oncogene has been reported by immune cells, such as monocytes (Ehrhard et al., 1993a) or T lymphocytes (Ehrhard et al., 1993b). It is therefore believed that neurotrophins, particularly NGF, may subserve important roles outside the nervous system.

These two lines of evidence, together with the structural homology of p75^{NGFR} to a series of surface receptors, including those for cytokines, and with the observed elevated plasma levels of NGF in patients with some autoimmune diseases (Dicou et al., 1993; Bracci-Laudiero et al., 1993; L. B.-L, L. A., E. G., and G. Rasi, unpublished data), led us to investigate whether NGF or its receptors or both were expressed by cells of the immune system. Here, we report that NGF is synthesized and released under basal conditions by normal human B lymphocytes, which also constitutively express both p75^{NGFR} and p140$^{trk\text{-}A}$ receptor chains. In addition, endogenous NGF functions in an autocrine fashion to maintain viability of cells with the surface phenotype of memory B cells, and its neutralization in vivo abrogates a secondary humoral immune response.

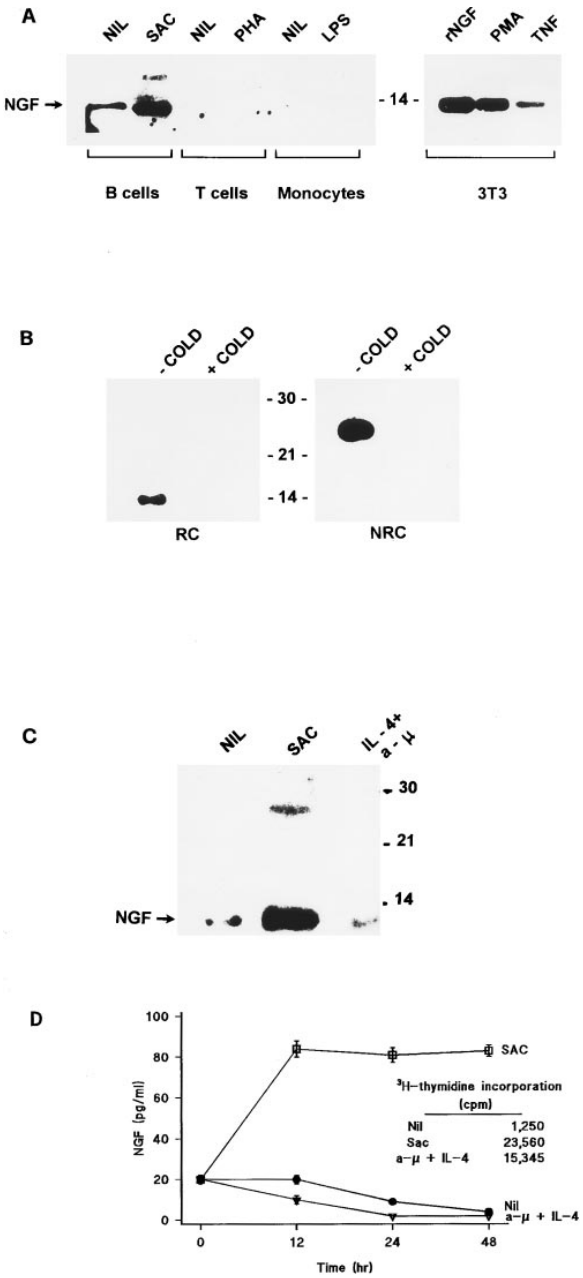

Figure 1. NGF Production by Normal Immunocompetent Cells

(A) Western blot analysis of culture supernatants from indicated human peripheral blood cells and mouse Swiss 3T3 fibroblasts as positive control. Human recombinant NGF was used as internal standard. Only B lymphocytes showed constitutive production of NGF, which increased after SAC stimulation.

(B) Endogenous labeling of peripheral blood B cells and NGF immunoprecipitation. Supernatants from ^{35}S-labeled B cells were immunoprecipitated with anti-NGF antibodies in absence (minus cold) or presence (plus cold) of excess unlabeled NGF and analyzed by 15% SDS–polyacrylamide gel electrophoresis under reducing (RC) and nonreducing conditions (NRC). Immunoprecipitable endogenously synthesized NGF was identified; detection of the NGF band was blocked by excess unlabeled NGF.

(C) Western blot analysis, immunostained with anti-NGF antibodies, of supernatants from 12 hr cultures of resting tonsil B lymphocytes, separated by centrifugation through Percoll density gradients and cultured with either no stimulus (NIL), SAC, or anti-μ plus IL-4. Only SAC increased NGF production.

(D) Time-course analysis of NGF production by resting tonsil B lymphocytes cultured with either no stimulus (closed circle), SAC (open box), or anti-μ plus IL-4 (open triangle). NGF concentration in culture supernatants was measured by ELISA. The value at time 0 shows the level of NGF in cell lysates of freshly isolated B cells. SAC stimulation induced increase in cytokine production, which in contrast gradually decreased in unstimulated or anti-μ plus IL-4–stimulated cultures. The same analysis performed on cell lysates revealed no NGF storage pool. The inset shows that resting B cells actively incorporated [^{3}H–]thymidine when stimulated with either SAC or anti-μ plus IL-4.

Results

NGF Production by Normal Human Immunocompetent Cells

During the course of a study to assess plasma levels of NGF in various pathological settings, we noticed that particularly high amounts of cytokine were present in patients with chronic liver diseases and other autoimmune conditions. To determine which cell type, if any, among immunocompetent cells was responsible for NGF production, normal peripheral blood or tonsil mononuclear cells were fractionated into T cells, B cells, and monocytes. These cells were then cultured in the presence or absence of relevant stimuli and subsequently tested for biochemical evidence of NGF synthesis and secretion. Only B lymphocytes constitutively produced NGF, and their generation of NGF was enhanced by stimulation with Staphylococcus aureus of the I Cowan strain (SAC) (Figure 1A). Immunoblotting demonstrated a major band in B lymphocyte–conditioned medium with anti-NGF antibodies (Figure 1A), but not with nonimmune immunoglobulin G (IgG) (data not shown). Immunoprecipitation of metabolically labeled B cell supernatants also showed a single major band with $M_r \approx 13$ kDa under reducing conditions or with $M_r \approx 26$ kDa under nonreducing conditions, the presence of which was blocked by inclusion of excess unlabeled NGF (Figure 1B). Kinetic studies of B cell lysates and supernatants showed no NGF storage pool (data not shown). In contrast with these results with B cells, no NGF production was observed with T lymphocytes or monocytes, even after stimulation (Figure 1A). Although it has been reported quite recently that some T cell clones can produce NGF (Ehrhard et al., 1993b), we became convinced that such cells are scarcely represented in a normal peripheral blood or tonsil sample (based on analysis of cell populations from at least twenty different donors), and we therefore focused on B cell production.

B lymphocytes were fractionated on density gradients, and high and low density fractions were studied as representative of resting and in vivo activated cells, respectively. Both B cell populations produced and efficiently released NGF (the latter approximately 60% more than the former; data not shown). Next, resting B cells were stimulated with antibodies to the human immunoglobulin μ chain constant region plus interleukin-4 (anti-μ plus IL-4) or with SAC, two stimuli active on B cells; the former activating surface IgM^{+} cells only, and the latter activating virtually all B lymphocytes (Romagnani

et al., 1982). It was evident that anti-μ plus IL-4 stimulation was ineffective, while SAC greatly enhanced NGF synthesis and secretion (Figure 1C). Analysis of supernatants of similar cultures by enzyme-linked immunoabsorbent assay (ELISA) confirmed that anti-μ plus IL-4–stimulated cells and unstimulated cells gradually reduced NGF production, while SAC-stimulated cells strongly increased it (Figure 1D). In contrast, both stimuli were effective with respect to induction of proliferative stimulation indices of greater than 10 and greater than 20, respectively (Figure 1D, inset). These findings indicated that SAC, but not anti-μ plus IL-4, triggered a metabolic pathway leading to NGF production. An alternative explanation was that lymphocytes normally responding to anti-μ plus IL-4, i.e., mostly virgin $s\mu^+\delta^+$ cells, did not alter NGF production (or possibly failed to produce it) following surface immunoglobulin (sIg) cross-linking, while the lymphocytes responding to SAC, a population also comprising cells with $s\gamma^+$ or $s\alpha^+$ ($s\gamma^+/\alpha^+$) phenotype, did, suggesting that the cytokine was involved in functional programs specific to the latter cell type.

Expression of NGF Receptor Molecules by Normal Human Immunocompetent Cells

The observation that NGF was produced by B lymphocytes prompted us to ascertain whether NGF receptors were also present, making possible an autocrine feedback loop. To this purpose, tonsil or peripheral blood T cells, B cells, and monocytes were studied for the expression of the two NGF binding molecules that are displayed by cells in the nervous system, the $p140^{trk\text{-}A}$ (trk) and the $p75^{NGFR}$. Western blot analyses of cell lysates using specific antibodies showed that each of the cell types studied expressed the trk molecule (Figure 2A); interestingly, $p75^{NGFR}$ was produced by B cells only (Figure 2B). Moreover, the same cells were studied by FACS following staining with antibodies to the $p75^{NGFR}$ chain and with Tmg 13.1, a recently described monoclonal antibody (MAb) to the extracellular cytokine binding region of the trk molecule (Eager, 1991). Consistent with the immunochemical approach, double staining was observed on B cells only, while T cells and monocytes were solely stained by Tmg 13.1 (data not shown).

The simultaneous expression of both receptor chains by B lymphocytes, a situation typical of nervous cells, indicated that these cells were equipped to respond to the full range of signals conveyed by the cytokine. We therefore concentrated on B cells, with the goal of obtaining data relevant to the functional role of NGF in lymphocytes.

To demonstrate binding of the cytokine, and to analyze its functional effects, we separated tonsil B lymphocytes into small and large cells and studied them for their ability to bind and internalize NGF, using equilibrium binding assays. Both resting and in vivo activated B cells efficiently internalized ^{125}I–NGF (data not shown). Figures 3A and 3B show the saturation curves of ^{125}I–NGF and their Scatchard transformations obtained with large and resting B cells. Large cells expressed, as expected, two classes of binding sites, demonstrating $K_d \approx 30$ pM (3×10^4 sites per cell) and $K_d \approx 1$ nM (10^6 binding sites per cell), respectively, consistent with the data reported on nervous cells. Surprisingly, when the same analysis was performed on small, resting B cells, no saturable binding could be observed, even upon incubation with very high ^{125}I–NGF concentrations (data not shown), in spite of the evident expression of both receptor chains (see Figure 2) and the efficient internalization of the cytokine. This marked discrepancy led us to consider the possibility that the endogenous ligand was actually occupying the receptor sites, a situation frequently encountered when autocrine circuits are acting (Cozzolino et al., 1989, 1990). Thus, purified small B

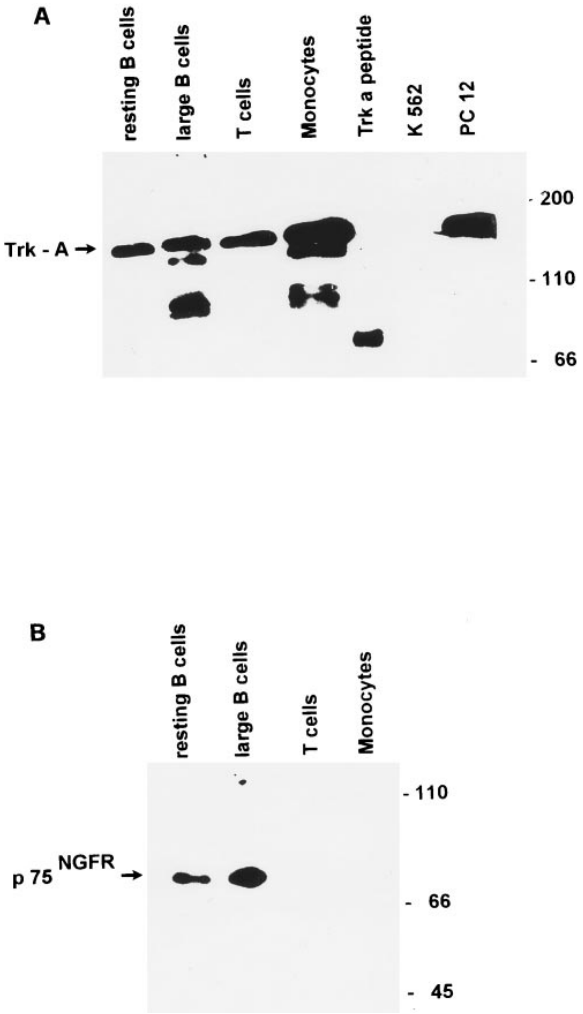

Figure 2. Expression of High Affinity ($p140^{trk\text{-}A}$) and Low Affinity ($p75^{NGFR}$) NGF Receptors by Normal Immunocompetent Cells

(A) Western blot analysis and immunostaining with anti-trk antibodies of lysates from resting B cells, in vivo activated (large) B cells, T cells, and monocytes. The trk peptide used for rabbit immunization and lysates from PC–12 cell line were used as positive controls; lysates from K562 cell line were used as negative control.

(B) Western blot analysis and immunostaining with anti-$p75^{NGFR}$ antibodies of lysates from resting B cells, in vivo activated (large) B cells, T cells, and monocytes.

High affinity NGF receptors were expressed by all immunocompetent cell types, while low affinity NGF receptors were expressed by B lymphocytes only.

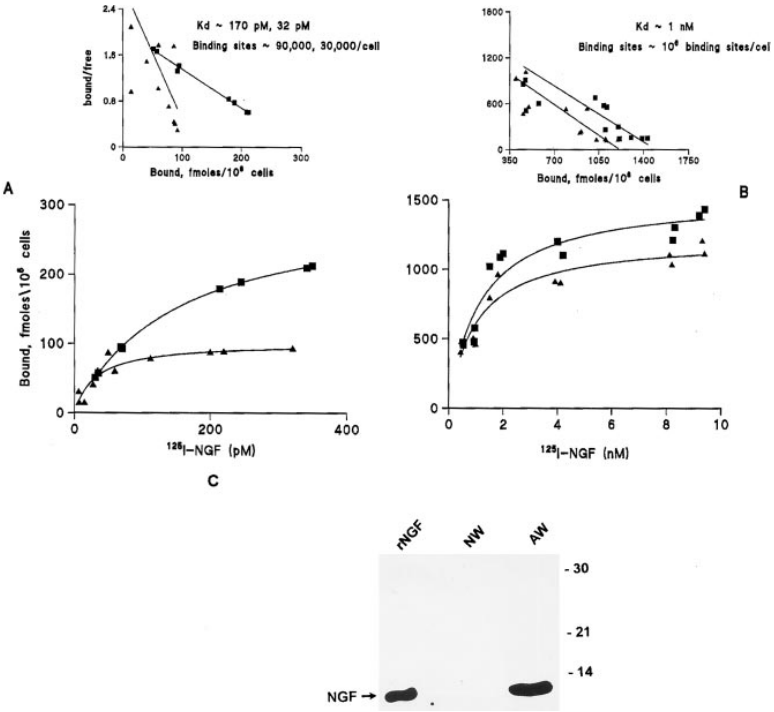

Figure 3. NGF Binding to B Lymphocytes

(A and B) Resting (closed box) or in vivo activated (open triangle) B cells were incubated with two ranges of ^{125}I–NGF concentrations in order to saturate high and low affinity NGF receptors.

(A) With NGF concentrations ranging between 10–400 pM, high affinity receptors were evident on both resting ($K_d \approx 170$ pM) and in vivo activated lymphocytes ($K_d \approx 30$ pM), with approximately 90,000 and approximately 30,000 binding sites per cell, respectively.

(B) High affinity receptors were detected on resting B lymphocytes after acidic treatment only. With NGF concentrations ranging between 0.5–10 nM, low affinity receptors were evident on both resting (closed box) and large B lymphocytes (open triangle), with a K_d of approximately 1 nM and approximately 10^6 binding sites per cell on both cell types.

(C) Endogenous NGF is tightly bound to surface NGF receptors. Resting tonsil B cells were washed with medium at pH 7.4 (NW, neutral wash) or with medium buffered at pH 3.0 (AW, acidic wash). The eluted proteins were TCA concentrated, blotted, and immunostained with anti-NGF antibodies. Human rNGF was used as positive control.

lymphocytes were briefly (60 s at 4°C) treated with culture medium buffered at pH 3.0 and then washed with regular medium before the binding assay. Under these conditions, both high and low affinity ($K_d \approx 170$ pM and 1 nM, respectively) receptors, with 9×10^4 and 10^6 binding sites per cell, respectively, could be detected (Figures 3A and 3B). To strengthen further the hypothesis that these sites were indeed occupied by NGF because of the existence of an autocrine loop, the acidic pH eluates (and the neutral pH eluates, as controls) were run in SDS–polyacrylamide gel electrophoresis, blotted, and stained with specific anti-NGF antibodies. As predicted, the cytokine was detected in the acidic eluates only (Figure 3C). Altogether, these results confirmed the expression of two classes of fully functional NGF receptors by B lymphocytes.

Effect of Endogenous NGF Neutralization in B Lymphocytes

The above data, indicating that B cells produced NGF and expressed high and low affinity receptors, supported the hypothesis of an autocrine circuit. To understand the function exerted by this circuit, we neutralized endogenous NGF and assessed its impact on properties of B lymphocytes. To this purpose, we employed either neutralizing antibodies to NGF or, in selected experiments, NGF antisense oligonucleotides. We first tested anti-NGF antibodies in conventional [^{3}H–]thymidine incorporation assays, using resting peripheral blood or tonsil B lymphocytes, stimulated with anti-μ plus IL-4 or with SAC. Both stimuli induced a vigorous response in the presence of control preimmune antibodies; in the presence of anti-NGF antibodies, the response to anti-μ plus IL-4 was unaffected, while that to SAC was diminished by 20%–30% (Table 1), initially suggesting that NGF was involved in the proliferative response to SAC. However, addition of exogenous recombinant NGF failed to increase [^{3}H–]thymidine incorporation (Table 1),

Table 1. Effect of Anti-NGF Antibodies on Mitogen-Induced Proliferation of Resting B Lymphocytes

	^{3}H-thymidine incorporation (cpm)		
	Exp. 1	Exp. 2	Exp. 3
UF B cells	251	240	306
UF B cells + NGF	305	312	403
UF B cells + SAC	13,808	32,010	17,040
UF B cells + SAC + anti-NGF	11,488	25,432	13,050
UF B cells + SAC + goat IgG	13,765	32,456	17,321
UF B cells + SAC + NGF	14,056	34,021	19,028
UF B cells + IL-4 + anti-μ	12,896	15,675	11,366
UF B cells + IL-4 + anti-μ + anti-NGF	12,798	15,254	10,987
UF B cells + IL-4 + anti-μ+goat IgG	12,913	16,070	11,654
UF B cells + IL-4 + anti-μ + NGF	13,004	16,876	12,114
Purified s$\mu^+\delta^+$ cells	384	426	368
Purified s$\mu^+\delta^+$ cells + SAC + goat IgG	6,863	7,121	7,256
Purified s$\mu^+\delta^+$ cells + SAC + anti-NGF	6,943	6,889	7,124
Purified sγ^+/α^+ cells	279	344	368
Purified sγ^+/α^+ cells + SAC + goat IgG	13,935	14,759	13,157
Purified sγ^+/α^+ cells + SAC + anti-NGF	2,468	3,137	2,324

Resting unfractionated (UF) B lymphocytes or the indicated purified populations were cultured for 48 hr and pulsed with ^{3}H-thymidine in the last 12 hr. Data are expressed as mean ^{3}H-thymidine incorporation of triplicate cultures. Standard deviation was always less than 10%. SAC was used at the final dilution of 1:10,000. Goat anti-NGF antibodies or preimmune goat IgG were used at the concentration of 10 μg/ml. Recombinant human NGF was used at the concentration of 100 ng/ml. IL-4 was used at the concentration of 100 U/ml. Polyclonal rabbit anti-μ was used at the concentation of 1 μg/ml.

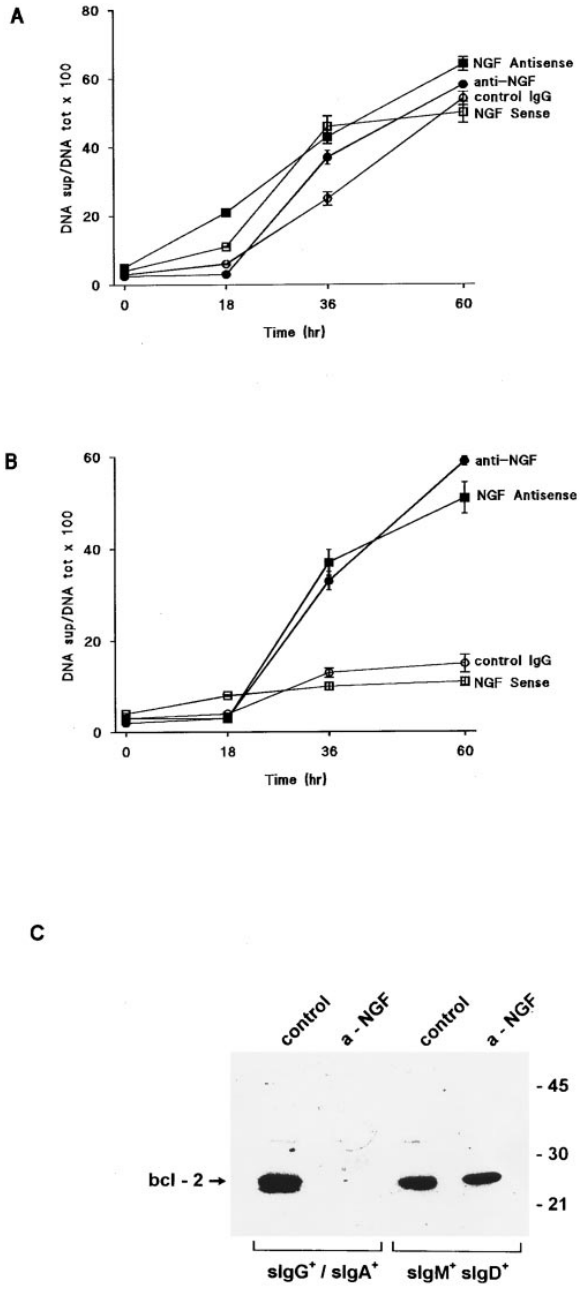

Figure 4. Effect of Anti-NGF Antibodies or Oligonucleotides on Purified B Cell Populations

(A and B) Resting $s\gamma^+/\alpha^+$ and $s\mu^+\delta^+$ B cells, purified by panning procedures, were cultured in the presence of anti-NGF antibodies (closed circle), control IgG (open circle), NGF antisense (closed box), or control sense (open box) oligonucleotides. At the indicated times, cells were lysed, and both nuclear and cytosolic DNA stained by diphenylamine reagent. In $s\mu^+\delta^+$ B cells, anti-NGF antibodies or NGF antisense oligonucleotides did not affect DNA fragmentation kinetics (A), which was accelerated by NGF neutralization in $s\gamma^+/\alpha^+$ B cells (B).

(C) Bcl-2 turnover in resting $s\gamma^+/\alpha^+$ and $s\mu^+\delta^+$ B cells following incubation with anti-NGF antibodies. Resting B cells, purified by panning procedures, were cultured in the presence of 10 μg/ml of goat anti-NGF antibodies or of preimmune goat IgG (control) for 18 hr. At the end of incubation, cells were lysed, blotted on nitrocellulose, and immunostained with anti–Bcl-2 MAbs. Bcl-2 protein completely disappeared in $s\gamma^+/\alpha^+$ cells cultured with anti-NGF antibodies but not with preimmune IgG. In contrast, anti-NGF antibodies did not affect Bcl-2 in $s\mu^+\delta^+$ B cells.

even in the presence of suboptimal doses of stimulants (data not shown), rather suggesting that the cytokine was not acting as a growth factor. Consistently, spontaneous proliferation of low density in vivo preactivated B cells was not modulated by anti-NGF antibodies (data not shown). These stimulation experiments with anti-μ plus IL-4 or SAC again indicated that different cell populations were responding to the mitogens and that they could be functionally divided on the basis of NGF utilization, in addition to NGF production (see also Figure 1C). To gain support for this concept, we separated resting tonsil B lymphocytes by panning into $s\mu^+\delta^+$ cells and $s\gamma^+$ and $s\alpha^+$ cells ($s\gamma^+/\alpha^+$), which were then stimulated with SAC or anti-μ plus IL-4. Table 1 shows that proliferation of $s\mu^+\delta^+$ cells in response to SAC was not affected by neutralizing anti-NGF antibodies. As expected, proliferation of $s\gamma^+/\alpha^+$ cells to anti-μ plus IL-4 was very weak (data not shown), while these cells were quite responsive to SAC; when stimulation was exerted in the presence of anti-NGF antibodies, [^{3}H–]thymidine incorporation was reduced by more than 80% ($p < 0.0001$). This finding, considered together with the results of the experiments on NGF production (see Figures 1C and 1D), suggested that the cytokine was critical for $s\gamma^+/\alpha^+$ cells and apparently dispensable for $s\mu^+\delta^+$ cells.

These data led us to explore further the effects of NGF on resting $s\mu^+\delta^+$ cells and $s\gamma^+/\alpha^+$ cells. Taking into account that it failed to increase in vitro growth of B cells (Table 1) and in view of the known property of NGF to maintain survival of neuronal cells (in its absence, those cells undergo apoptosis), we considered whether NGF might enhance survival of resting $s\mu^+\delta^+$ cells or $s\gamma^+/\alpha^+$cells. Thus, the above populations, purified from tonsils, were cultured in the presence of anti-NGF antibodies or NGF antisense oligonucleotides for various time intervals, and the ratio of fragmented to intact DNA, as a measure of the percentage of cells undergoing apoptosis, was recorded. When $s\mu^+\delta^+$ cells were analyzed, equal proportions of cells were apoptotic, both those treated with anti-NGF reagents and those with control preimmune IgG (or sense oligonucleotide) (Figure 4A). In contrast, cultures of $s\gamma^+/\alpha^+$ cells treated with anti-NGF antibodies or oligonucleotides contained more than 60% apoptotic cells at 60 hr, whereas control cultures had less than 20% apoptotic cells ($p < 0.0001$) (Figure 4B); interestingly, the kinetics of apoptosis were much slower compared with that of $s\mu^+\delta^+$ cells, suggesting an important difference in the life potential between the latter cells and $s\gamma^+/\alpha^+$ cells in vitro.

On the whole, this set of experiments demonstrated that endogenous NGF was an autocrine survival factor for $s\gamma^+/\alpha^+$ cells, since its neutralization triggered their apoptotic death, but not for $s\mu^+\delta^+$ cells, suggesting that only the former cells could elaborate the cytokine. To test this concept, purified populations were analyzed for NGF production by ELISA. It was evident that $s\gamma^+/\alpha^+$ cells produced at least 8-fold more NGF than $s\mu^+\delta^+$ cells (409 $\pm$ 19 pg/10^7 cells versus 51 $\pm$ 4 pg/10^7 cells; $n = 9$).

Thus, to ascertain whether the functional difference between the subsets was due to utilization, rather than production, of NGF, we investigated an important determinant of the pathway leading to apoptosis, Bcl-2 protein turnover (Korsmeyer, 1992). Purified resting $s\gamma^+/\alpha^+$ and $s\mu^+\delta^+$ populations were cultured with anti-NGF or control antibodies for 18 hr and then lysed to analyze their intracellular content of Bcl-2 protein. Figure 4C shows that neutralization of endogenous NGF caused the complete disappearance of Bcl-2 protein from $s\gamma^+/\alpha^+$ cells but did not affect Bcl-2 protein content in $s\mu^+\delta^+$ cells, revealing a major difference between the subpopulations with respect to NGF utilization.

Anti-NGF Antibodies Deplete Memory B Cells In Vivo

Since $s\mu^+\delta^+$ phenotype identifies virgin B cells, whereas cells with $s\gamma^+/\alpha^+$ phenotype comprise those lymphocytes that have already undergone the process of immunoglobulin constant region class switch, including memory B lymphocytes (Kishimoto and Hirano, 1989; Sprent, 1994), we hypothesized that the autocrine NGF served as a survival factor for memory B cells. We decided to test this concept, using an in vivo approach to analyze the well-defined hapten-specific systems Nitrophenol–bovine serum albumin (NIP–BSA) or Arsonate–keyhole limpet hemocianin (Ars–KLH), or the complex (mosaic) antigen system tetanus toxoid (TT). First, we demonstrated a substantial identity between human and murine B cells regarding NGF synthesis (murine $s\gamma^+/\alpha^+$ and $s\mu^+\delta^+$ cells constitutively produced 234 $\pm$ 21 pg/10^7 and 28 $\pm$ 3 pg/10^7 cells, respectively), NGF receptor expression (murine unfractionated sIg^+ cells expressed approximately 4,800 high affinity [$K_d \approx 135$ pM] and approximately 10^6 low affinity [$K_d \approx 1.1$ nM] binding sites per cell), and functional significance of the cytokine for cell survival in vitro ($s\gamma^+/\alpha^+$ cells presented approximately 70% DNA fragmentation upon exposure to neutralizing anti-NGF antibodies). Thus, groups of twenty BALB/c or C57BL/6 mice were immunized with the relevant antigen, and after 40 days a group of ten animals received a single dose of neutralizing anti-NGF IgG, while the other ten mice were injected with nonimmune IgG as control. After an additional 48 hr, all animals received a recall dose of the respective antigen; 4 days later, all mice were sacrificed, and plasma concentrations of antigen-specific IgM and IgG were determined by ELISA. Animals receiving anti-NGF antibodies showed levels of specific IgG to the immunogen markedly lower than those of controls ($p < 0.001$) (Figure 5A). In contrast, no difference was observed in the concentration of antigen-specific IgM (Figure 5B), indicating that the antibody response generated by the newly formed virgin B lymphocytes encountering the immunogen, the second primary response, was not affected. To strengthen further the latter conclusion, two additional groups of mice were first treated with anti-NGF IgG or with control IgG, then (48 hr later) immunized with TT, and after 1 week sacrificed to determine their plasma level of TT-specific IgM, which was not statistically different in the two populations (0.275 $\pm$ 0.032 absorbance 405 nm arbitrary units in treated animals versus 0.284 $\pm$ 0.041 in controls) (data not shown).

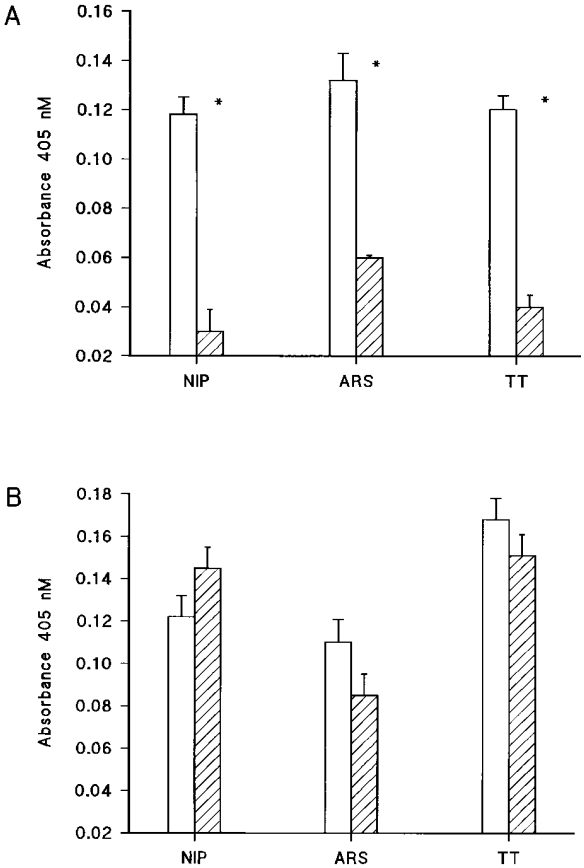

Figure 5. Reduction of Antigen-Specific IgG Titer Induced by In Vivo Treatment with Anti-NGF Antibodies

We immunized three groups of twenty mice with NIP–BSA, Ars–KLH, or TT. After 40 days, ten mice of each group were treated with anti-NGF antibodies (striped box), the others with preimmune IgG (open box); 48 hr later, all animals were injected with a recall dose of the relevant antigen. Serum levels of NIP-, Ars-, and TT-specific IgG (A) and IgM (B) were measured by ELISA 4 days after the recall injection. The mean absorbance of serum samples in each group before the antigen boost was subtracted from the values recorded at the end of the experiments (*$p < 0.001$).

The above findings suggest that, consistent with in vitro data, anti-NGF antibodies were able to induce cell death of most isotypically switched memory B cells, thus quenching a secondary humoral response. To obtain direct evidence supporting this concept, we treated two groups of normal adult mice with anti-NGF IgG or with control IgG. After 3 days, spleen cells were isolated, and the proportions of B cell subpopulations were assessed by cytofluorimetry. Figure 6 shows that the percentage of $s\gamma^+/\alpha^+$ cells was markedly reduced in spleens of treated mice, compared with controls, while no significant difference between the two groups was observed in the percentage of $s\mu^+\delta^+$ cells. On the whole, this set of experiments indicates that NGF in vivo also plays a role in the maintenance of memory B cells.

NGF Is Not a Switch Factor for B Lymphocytes

The outcome of the above in vivo NGF neutralization experiments was compatible with the hypothesis that

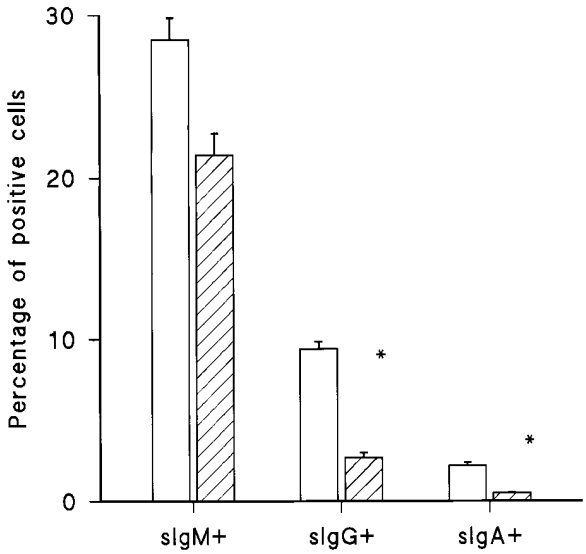

Figure 6. In Vivo Effect of Anti-NGF Antibodies on Mouse Spleen B Cell Subpopulations

Splenocytes from normal adult mice treated with neutralizing anti-NGF antibodies (1 mg/mouse) (striped box) or with preimmune goat IgG (1 mg/mouse) (open box) were immunostained with antibodies specific for the Fc region of mouse Ig μ, Ig γ, or Ig α chain, and analyzed by flow cytometry. Percentage of $s\gamma^+$ or α^+ B cells was strongly reduced in mice treated with anti-NGF, compared with mice treated with preimmune IgG. (*$p < 0.001$)

the cytokine was an autocrine survival factor for memory B lymphocytes. However, the same results would have been observed if NGF were involved in the machinery that governs the IgM→IgG class switch, along with the CD40 ligand (Aruffo et al., 1993). If this is true, however, a higher proportion of IgM-producing cells, together with a reduced amount of IgG- or IgA-producing cells, should be observed whenever a polyclonal population of lymphocytes is induced to differentiate in vitro in the presence of anti-NGF antibodies. Thus, normal human peripheral blood mononuclear cells were cultured for 10 days with pokeweed mitogen to stimulate polyclonal differentiation (Kishimoto and Hirano, 1989), and with either anti-NGF IgG or nonimmune IgG as negative controls, or with soluble CD40 pentamer, a genetically engineered molecule that prevents the CD40–gp39 interaction (Fanslow et al., 1992) as a positive control. Immunoglobulin measurement by ELISA revealed that anti-NGF antibodies induced a definite reduction in the level of IgG and IgA in the pokeweed mitogen-stimulated cultures, but also IgM levels approximately 20% lower than those of control cultures (Table 2). By contrast, the soluble CD40 pentamer caused a depression of IgG and IgA secretion compared with controls and an increase in the rate of IgM secretion (Table 2).

These experiments indicated a clear difference in the mechanisms likely to be responsible for the observed changes. In fact, the soluble CD40 pentamer caused a block in the immunoglobulin class switch phenomenon, inducing an "accumulation" of cells in the preswitch (i.e., $s\mu^+$) compartment; in contrast, anti-NGF antibodies caused only a strong reduction of IgG- and IgA-producing cells, an evidence of disappearance (death) of the

Table 2. Effect of Anti-NGF Antibodies on Ig Production by PWM-Stimulated PBMNC

	Ig (ng/ml)		
	IgM	IgG	IgA
Medium alone	84	30	676
PWM	1,028	966	10,716
PWM + a-NGF	674	374	1,350
PWM + goat IgG	987	995	9,243
PWM + CD40μ	1,530	554	4,794
PWM + CD4μ	928	1,024	9,865

Human PBMNC were cultured at 3×10^6 cells/ml for 12 days in the presence or absence of PWM (10 μg/ml), goat anti-NGF antibodies or preimmune goat IgG (10 μg/ml), CD40μ or CD4μ supernatant (1:10 final dilution). At the end of the incubation, supernatants were collected and IgA, IgG, and IgM were measured by ELISA using specific antibodies. Data are expressed as mean Ig concentration of triplicate wells; standard deviation was less than 15%.

respective precursors, induced by anti-NGF antibodies. These data are consistent with the results of the above in vivo experiments, whereby the reduction of $s\gamma^+$ or $s\alpha^+$ cells was not accompanied by an increase of $s\mu^+$ cells. As a whole, these findings strongly indicate that NGF was not acting as a "switch factor" for normal human or murine B lymphocytes.

Discussion

The ability to discriminate finely among a multitude of chemical structures and to retain a trace of these encounters (specificity and memory) are the hallmarks of the immune system. While the molecular bases of specificity have been largely defined through the extensive characterization of the biochemistry and genetics of the antigen receptors expressed by T and B lymphocytes, knowledge of immune memory is still largely phenomenological. In particular, it remains to be defined whether a single memory cell, like most lymphocytes, has a definite and limited life span in quiescent conditions or is a long-living cell lasting for years, possibly throughout life. Several hypotheses have been proposed, the prevailing one being that the long persistence of antigen within lymphoid organs results in a continuous slow proliferation of immune cells that maintain immunological memory (Gray, 1993). However, it is difficult to imagine how antigens can remain unmodified for years, particularly if proteinaceous (Sprent, 1994). In fact, evidence has been provided that immunological memory can be maintained for quite long periods by noncycling cells (Schittek and Rajewsky, 1990), although the molecular mechanisms allowing their survival are still unknown. In the present paper, we show evidence suggesting that memory B lymphocytes, when generated, may undergo the same fate as Bizzozzero "perennial" cells, whose prototypes are neurons, because of their ability to produce an autocrine survival factor, NGF.

The major argument suggesting that the cytokine acts as a survival rather than a growth factor is based on the observation that preventing NGF receptor triggering of purified $s\gamma^+/\alpha^+$ resting B cells by neutralizing anti-NGF antibodies caused massive cell death by apoptosis. In

addition, when resting unfractionated B cells were stimulated with anti-μ antibodies plus IL-4, which elicited a strong proliferative response, they did not increase NGF production rate and their proliferation was not augmented by exogenous recombinant NGF, nor was it affected by anti-NGF antibodies. Moreover, when SAC was used to stimulate the same cells, increased amounts of NGF were secreted, but further addition of exogenous cytokine failed to potentiate [^{3}H–]thymidine incorporation, even if suboptimal doses of stimulant were applied. Consistently, low density in vivo preactivated B cells expressed more NGF, but saturating doses of anti-NGF antibodies did not abolish B cell proliferation, as would be expected if the cytokine were a growth factor. Taken together, these considerations instead pointed to NGF as a survival factor.

This conclusion might appear to conflict with reports that in the past years have suggested an intrinsic growth-stimulating activity of NGF on lymphocytes (Otten et al., 1989; Brodie and Gelfand, 1992). However, these reports can be reinterpreted taking into account that, according to our findings, addition of exogenous NGF may reduce the number of some cells spontaneously dying in vitro (particularly those originating IgA- and IgG4-secreting cells; Kimata et al., 1991) because of low density culture conditions, eventually leading to a higher basal proliferation in culture. Moreover, distinction between growth and survival factor may be purely nominalistic, as suggested by the observation that IL-3 and stem cell factor support long-term survival of dormant noncycling lymphohematopoietic progenitors in liquid cultures (Katayama et al., 1993).

The finding of spontaneous production of NGF by B cells lends further support to the evidence of a phlogistic role of NGF (Aloe et al., 1994), which probably contributes in a paracrine fashion to the function of inflammatory cells, such as macrophages, which are equipped with the receptor machinery necessary to respond to the cytokine (Ehrhard et al., 1993a) but unable to produce it (Santambrogio et al., 1994; Figure 1B, our data). Similar considerations apply for T lymphocytes. In this study, we make no attempts to characterize more precisely, from a phenotypic or functional standpoint, the (sub)populations of T cells expressing NGF receptors and hence probably responding to it. However, B cell–derived NGF might subserve important roles in the complex event of B cell antigen presentation to T lymphocytes, particularly when a secondary immune response has to take place. In fact, sIgG$^+$ memory B cells express receptors with the highest affinity for the antigen (Siekevitz et al., 1987; MacLennan and Gray, 1986) and are therefore privileged in the competition for binding that occurs when the antigen is in limited amounts. Their ability to release NGF might be essential for a proper activation of either memory or naive T cells.

An important point that has emerged from our study is the simultaneous expression of both chains, the trk molecule and the p75^{NGFR} molecule, known to bind NGF by B lymphocytes, a feature typically presented by neuronal cells. Although NGF was the first cytokine to be characterized, relatively little still is known about the specific metabolic pathways triggered by its interaction with either receptor chain, nor is it clear whether or not both chains cooperate in binding a single molecule of ligand, as conflicting results on this topic have been reported (Jing et al., 1992; Benedetti et al., 1993; Huber and Chao, 1995). However, it has been shown that expression of p75^{NGFR} induced neural cell death constitutively when the protein was unbound, while its binding by NGF or by monoclonal antibodies inhibited cell death (Rabizadeh et al., 1993), suggesting that it is, per se, able to transmit a biological signal, consistent with the structural homology between p75^{NGFR} and a series of receptor chains, including TNFRI, TNFRII, Fas/Apo-1, and CD40, all involved in inducing or preventing apoptosis in target cells (reviewed by Raffioni et al., 1993). These latter data are entirely in accord with our finding that neutralization of the autocrine NGF, but not treatment with anti-p75^{NGFR}, determines B cell death (unpublished data), pointing to this receptor chain as a mediator of signals acting upon the survival–death divide. Interestingly, in spite of the structural homology between p75^{NGFR} and CD40, it is clear that NGF does not take part in the immunoglobulin class switch process.

In some cell types, such as keratinocytes or melanocytes (Yaar et al., 1994; Di Marco et al., 1993), trk mediates signals that potentially stimulate cell proliferation, in contrast with what occurs in neuronal cells, whereby NGF prevents apoptosis following trk engagement, possibly via phosphatidylinositol-3 kinase activation (Yao and Cooper, 1995). This discrepancy might be solved by the existence of another chain or chains that may participate in the formation of a multichain receptor complex with trk or p75^{NGFR} or both, as usually occurs for most cytokine receptors. The latter hypothesis would also explain the limited but significant difference we observed in NGF binding affinities displayed by large and resting B cell populations (approximately 30 pM versus approximately 170 pM, respectively; see Figure 4).

Separation of normal resting B lymphocytes on the basis of surface immunoglobulin isotype expression identifies two functionally different subpopulations; i.e., s$\mu^+\delta^+$ virgin and sγ^+/α^+ memory cells (Kishimoto and Hirano, 1989). We used this approach to gain insights into the role of NGF autocrine circuit and observed several features that led us to suggest that the cytokine is an endogenous survival factor for memory cells, both in humans and in mice. First, while both virgin and memory cells expressed basically the same levels of NGF receptors, the latter produced at least 8-fold more NGF protein than the former. Second, treatment with neutralizing anti-NGF antibodies induced disappearance of Bcl-2 protein and, consistently, massive DNA fragmentation in sγ^+/α^+ cells, while it was insignificant for s$\mu^+\delta^+$ cells. Third, in vivo administration of anti-NGF antibodies abolished secondary antigen-specific immune responses but failed to affect the primary IgM response. Finally, a single injection of anti-NGF antibodies to normal animals caused a marked reduction in the percentage of isotypically switched B lymphocytes, which comprise memory cells. On the other hand, these findings raise at least two important issues, namely, the maturational stage at which NGF gene expression takes place and the relationships between NGF and Bcl-2.

The low but detectable production of NGF by s$\mu^+\delta^+$

cells would indicate a relatively early onset during B cell ontogeny, whose functional significance should be further elucidated. However, based on quantitative considerations about the magnitudes of different B cell subpopulations, it was suggested that $s\mu^+$ cells also comprise memory lymphocytes, able to originate cells secreting immunoglobulin other than IgM (Gray, 1993). If so, the $s\mu^+$ cell-derived NGF we observed could be produced by the latter cells and, in this case, NGF gene expression might be linked to, and possibly regulated by, the same molecular mechanisms operating the immunoglobulin class switch.

Little is presently known about the molecular pathways that relate NGF receptor chains to Bcl-2, a protein critically regulating survival both in neurons and in lymphoid cells, particularly in memory B cells (Batistatou et al., 1993; Hawkins and Vaux, 1994; Nunez et al., 1991), which in fact disappears from resting $sIgG^+$ or $sIgA^+$ cells treated with neutralizing anti-NGF antibodies. Recently, evidence has been provided that reactive oxygen species and, more generally, alterations in the cellular redox potential may be involved (Greenlund et al., 1995). In this connection, it has been shown that low-rate nitric oxide production in Epstein–Barr virus-infected B lymphocytes, constitutively expressing nitric oxide synthase, prevents apoptosis (Mannick et al., 1994), probably acting at multiple levels on thiol-sensitive pathways that also regulate metabolism of Bcl-2, susceptible to intracellular redox potential and participating in its maintainance (Hockenbery et al., 1993). Interestingly, Mosialos et al. (1995) quite recently showed that functional relationships exist among the proteins that transduce signals from the TNF–Fas–NGF receptor family; since TNF induces alterations in cellular redox equilibrium (Ishii et al., 1992), these molecules could also participate in the adaptive response elicited by NGF. We are presently investigating the latter hypothesis in memory B cells following NGF neutralization.

One striking piece of evidence deserving consideration is the observation that, in lymphoid cells at least, both apoptosis and cell survival are regulated through autocrine circuits, the former involving Fas/Apo-1 and its ligand (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995), the latter involving NGF, according to our data. Such an arrangement ensures that all the molecular requirements needed to commit the cell to either pathway are immediately available and helps us understand how lymphoid organs are sites in which complex functional changes may occur in an ordered manner. The evidence that the autocrine circuit is public (involving secretion of ligands and binding to extracellular receptors) instead of private, or intracrine, strongly suggests that function of both systems may be modulated by either receptor antagonists or soluble receptors, originating a complex network of "social" controls over the fate of a single lymphocyte. Interestingly, the latter complexity may yield several opportunities to interfere with such dynamics using pharmacological approaches.

Experimental Procedures

Cell Isolation and Culture

Human T lymphocytes were separated from peripheral blood mononuclear cells or tonsil cells by E-rosetting. Monocytes were isolated by adherence on plastic petri dishes. B lymphocytes were further purified from the non-T population using CD19-coated magnetic beads. Murine B lymphocytes, depleted of monocytes as above, were isolated from spleens by two rounds of negative selection using an anti-CD3ϵ MAb (Boehringer Mannheim). The purity of population was more than 95%, as assessed by flow cytometry. Human or murine B cells were further fractionated into resting (high density) and activated (low density) cells by Percoll density gradients. The proportions of $sIgM^+$, $sIgG^+$, and $sIgA^+$ cells in tonsil resting B cells were typically 65%, 25%, and 10%, respectively, as assessed by flow cytometry.

For proliferation assays, B cells were cultured in 96-well plates at a concentration of 10^6/ml in RPMI 1640 medium (GIBCO), supplemented with 10% (v/v) fetal calf serum (Hyclone), for 72 hr in humified air with 5% CO_2. Heat-inactivated SAC (Boehringer Mannheim) was used at the final dilution of 1:10,000. Human rIL-4 (R & D) was used at 100 U/ml, rabbit anti-human chain was used at 1 g/ml, NGF (Boehringer Mannheim) was used at 100 ng/ml, neutralizing goat anti-NGF antibodies (R & D; $ND_{50} = 10$ μg/ml in the IMR–32 neuroblastoma cell proliferation assay [Janet et al., 1995] in response to 100 ng/ml of NGF) or preimmune goat IgG were used at 10 μg/ml. Cells were pulsed with 0.5 μCi of [^{3}H–]thymidine in the last 12 hr of culture and counted in a β scintillation counter.

For immunoglobulin production, human peripheral blood mononuclear cells were cultured for 12 days in the presence or absence of pokeweed mitogen (10 μg/ml, GIBCO), anti-NGF antibodies (10 μg/ml), preimmune goat IgG (10 μg/ml), and CD40-μ or CD4-μ supernatant (1:10 final dilution). Supernatants were collected and tested for IgG, IgA, and IgM production by ELISA.

For analysis of NGF production, cells (2×10^7) were cultured at 10^7/ml in serum-free RPMI 1640, supplemented with 10% (v/v) Nutridoma (Sigma) for different times in the presence or absence of SAC (1:10,000 final dilution), phytohemoagglutinin (1 μg/ml, GIBCO), lipopolysaccharide (10 μg/ml, Sigma), and supernatants assayed by ELISA.

B cell subpopulations were isolated by panning procedures using plastic petri dishes coated with rabbit anti-human IgM and rabbit anti-human IgD (referred to as $s\mu^+\delta^+$), or rabbit anti-human IgG plus rabbit anti-human IgA (referred to as $s\gamma^+/\alpha^+$), or goat anti-mouse IgM and IgD or IgG and IgA, prepared as described in Wysocki and Sato (1978). The purity of the isolated population was always greater than 90%, as assessed by flow cytometry using specific MAbs. To rule out the interference of the activation process following sIg receptor triggering, all the experiments were repeated using the reciprocal nonadherent populations (e.g., $s\mu^-\delta^-$ cells, mostly comprising $s\gamma^+/\alpha^+$ cells), recovered after three rounds of adherence from immunoglobulin-coated plates.

Determination of Cell Viability and DNA Fragmentation

Human and murine B cell subpopulations, cultured at 5×10^6/ml with 10 μg/ml anti-NGF IgG or preimmune IgG, or with 20 μM 18-mer antisense or sense oligonucleotides complementary to the nucleotides 54–72 of the NGF coding region (Primm), were diluted 1:1.5 with 5 mM Tris, 20 mM EDTA, and 0.5% (v/v) Triton X–100 (pH 8.0) and allowed to lyse for 15 min on ice before centrifugation for 20 min at 27,000 $\times$ g, to separate intact chromatin (pellet) from DNA fragments (supernatant). Pellets were resuspended in 5 ml of a buffer containing 10 mM Tris and 1 mM EDTA (pH 8.0), and pellet and supernatant samples were assayed for DNA content, using the diphenylamine reagent (1.5% diphenylamine in acetic acid plus 10% acetaldehyde) for 16 hr at 30°C (Burton, 1956). The optical density at 600 nm was measured for each sample. Percentage of DNA fragmentation was calculated according to McConkey et al. (1989). Cell viability was evaluated by trypan blue dye exclusion.

Radioligand Binding Studies

For analysis of surface NGF receptors, human resting tonsil or murine splenic B lymphocytes were acid-treated with RPMI 1640 medium buffered at pH 3.0 for 1 min on ice and washed with phosphate-buffered saline (PBS). Acid-treated resting B lymphocytes and in vivo activated large B lymphocytes were then incubated at 10^6/ml with different concentrations of ^{125}I-NGF (Amersham; specific activity 50 μCi/g), in the presence or absence of excess unlabeled human

rNGF for 2 hr at 4°C. Cells were washed, and bound radioactivity was counted in a γ counter. Specific binding was calculated for each experimental point, and data were analyzed by a scientific program (Figure P, Biosoft). For radioligand internalization, cells were cultured as above with 0.5 nM ^{125}I-NGF in the presence or absence of excess unlabeled NGF, washed and cultured at 37°C for 2 hr, then treated with glycin buffer (pH 2.8) and lysed. Membrane-bound (acidic eluate) and cell-associated radioactivity were determined by counting in a γ-counter. The neutral pH wash and the acidic pH eluate from 10^8 resting B cells was TCA concentrated, blotted on nitrocellulose, and immunostained with anti-NGF IgG or with preimmune IgG for detection of the receptor-bound endogenous ligand.

Immunochemical Analysis

For Western blot analysis, supernatants or NP–40 (0.25% in PBS) lysates of 2×10^7 cells were TCA precipitated, washed with ethanol, and diluted 1:1 in 2-mercaptoethanol Laemmli buffer. Samples were run in SDS–polyacrylamide gel electrophoresis, blotted against nitrocellulose filters, and immunostained with the appropriate antibodies (goat anti-NGF, rabbit anti-*trk* [Genzyme], mouse anti-p75^{NGFR} [Boehringer Mannheim], mouse anti–Bcl-2 [Santa Cruz Technology]) or with preimmune immunoglobulin. The antigen–antibody complexes were visualized using appropriate secondary antibodies and the ECL detection system, as recommended by the manufacturer (Amersham).

For endogenous labeling and immunoprecipitation studies, cells were washed three times with cystein-free RPMI 1640 (GIBCO) and cultured at approximately 10^7/ml in the same medium supplemented with 5% dialysed fetal calf serum and 200 μCi of [^{35}S–]cystein (Amersham; specific activity 800 Ci/mM) for 4 hr at 37°C in 5% CO_2. Supernatants were removed, and cells were washed in cold PBS and lysed in 0.25% NP–40 plus 1 mM phenylmethylsulfonyl fluoride. Supernatants and cell lysates were immunoprecipitated as described in Cozzolino et al. (1990) in the presence or absence of 100 μg/ml of human rNGF. The immunoprecipitates were washed, eluted in SDS Laemmli buffer, with or without 2-mercaptoethanol, and run on 15% SDS–polyacrylamide gel electrophoresis slabs, which were treated with Amplify (Amersham), dried, and exposed to Hyperfilm MP (Amersham) at −70°C.

Mice Immunization and Treatment

Ars–KLH was prepared by diazoting p-aminophenylarsonic acid and coupling to KLH (Calbiochem) in a ratio of 40 mg of hapten to 1 g of protein (Nisonoff, 1967). (4-Hydroxy-5-iodo-3-nitro-phenyl)acetyl (NIP, a gift of Dr. D. Schilovich, HSR) was coupled to BSA (NIP–BSA) according to the procedure described by Reth et al., 1978. Two groups of twenty female BALB/c mice, 6 weeks old, were injected subcutaneously in the neck with 0.1 ml of TT solution (15 μg/ml [Anatetal, Biocine-Sclavo]) or intraperitoneally with Ars–KLH (0.1 mg), and a group of twenty C57BL/6 mice was immunized intraperitoneally with 0.1 mg NIP–BSA. We treated ten mice of each group forty days after priming with goat anti-NGF IgG (500 μg/mouse), the other with preimmune goat IgG (500 μg/mouse), and 48 hr later all mice were boosted with the same dose of the relevant antigen. Within 4 days after the second immunization, blood was withdrawn from the retro-orbital venus plexus. Other groups of ten mice immunized with TT, NIP–BSA, or Ars–KLH were used for the measurement of antigen-specific IgG immediately before the boost injection.

For FACS analysis of splenocytes, groups of ten adult BALB/c mice were treated with two injections in 72 hr (0.5 mg/mouse) of anti-NGF antibodies or goat IgG. After an additional 72 hr, mice were sacrificed and splenocytes, depleted of monocytes, were obtained. Percentage of surface IgG$^+$, IgA$^+$, or IgM$^+$ splenocytes was determined by cytofluorimetry using specific antibodies.

ELISA

NGF ELISA was performed as described by Söderström et al. (1990). For evaluation of TT-specific IgG or IgM titer, plates were coated with 50 μl of TT (10 μg/ml) in 0.1 M borate buffer (pH 8.6) overnight at 4°C. For evaluation of NIP or Ars-specific IgG or IgM titer, plates were coated with NIP–KLH or Ars–BSA (35 μg/ml in PBS). Specific IgG and IgM titer to the immunizing complexes were also evaluated by coating the plates with NIP–BSA or Ars–KLH. After saturation with PBS containing 1% BSA and rinsing with PBS with 0.05% (v/v) Tween 20, coated wells were incubated for 1 hr at 37°C with serial dilution of mouse sera; a pool of preimmune mouse sera was used as negative control. Bound immunoglobulin were detected by addition of alkaline phosphatase–conjugated goat anti-mouse IgG or IgM. The final reaction was visualized by incubating with NPP (Sigma) substrate solution, and absorbance at 405 nm was recorded.

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