highly homologous to that from mouse. Three immunizations of the rabbit were performed with 50 μg of purified neuroleukin emulsified in Freund's adjuvant. Complete adjuvant was used for the first immunization and incomplete adjuvant for the second and third immunizations. The emulsion was injected subdermally at multiple sites.
29. Synthesis of a single-stranded DNA probe was primed from the 3′ end of the neuroleukin coding sequence (cloned into M13mp9) nucleotides 1685 to 1699, with the oligonucleotide CTGTTGCTTGATGAA used at a 5:1 molar ratio. The synthesis was performed with the Klenow fragment of DNA polymerase I (New England Biolabs) and [α-^{32}P]dATP (410 Ci/mmol). M13 sequences were trimmed from the probe with Eco RI and then the probe was denatured and purified by electrophoresis through a low melting temperature agarose gel. Hybridization with the probe at 10^6 cpm was performed in 5× standard saline citrate (SSC), 1× Denhardt's solution, 0.1 percent SDS, and salmon sperm DNA (Sigma) (100 μg/ml) at 55°C. Filters were washed first in 5× SSC and 0.1 percent SDS at 55°C, then in 0.1× SSC and 0.1 percent SDS at 55°C. The filters were then exposed to Kodak XAR5 film at −70°C.
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Neuroleukin: A Lymphokine Product of Lectin-Stimulated T Cells

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Neuroleukin is a lymphokine product of lectin-stimulated T cells that induces immunoglobulin secretion by cultured human peripheral blood mononuclear cells. Neuroleukin acts early in the in vitro response that leads to formation of antibody-secreting cells, but continued production of immunoglobulin by differentiated antibody-secreting cells is neuroleukin-independent. Although the factor is not directly mitogenic, cellular proliferation is a late component of the response to neuroleukin. Neuroleukin does not have B-cell growth factor (BCGF) or B-cell differentiation factor (BCDF) activity in defined assays. Neuroleukin-evoked induction of immunoglobulin secretion is both monocyte- and T cell-dependent.

IMMUNE RESPONSES OF B LYMPHOCYTES, INCLUDING ACTIVATION, clonal expansion, antibody secretion, and immunoglobulin heavy chain class switching are regulated by an unknown number of T cell–derived lymphokines (*1*). Interleukin-1 (IL-1), interleukin-2 (IL-2), and γ-interferon (IFN-γ) all modulate B-cell function, and another dozen or so factors in T-cell or lymphoblastoid-cell supernatants have been partially characterized (*1, 2*). The T cell–derived regulators of B-cell immune responses are of particular interest because of their potential clinical use in treating humoral autoimmunity or immunodeficiency.

So far only a single T cell–derived lymphokine regulator of B-cell function has been characterized at the molecular level (*3*). The factor, termed IL-4 (*3*), elicits three different in vitro responses by purified mouse splenic B cells. IL-4 directly stimulates quiescent B cells to increase surface expression of Ia; it is mitogenic for B cells costimulated with antibody to immunoglobulin (Ig), and it induces a switch from synthesis of IgG3 to IgG1 by B cells costimulated with lipopolysaccharide (LPS), a T cell–independent, polyclonal B-cell activator. The B-cell responses measured in the three in vitro assays are so different that IL-4 was earlier thought to be three different lymphokines (*3–5*).

That a single lymphokine acting on B cells can have such

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divergent biological effects was unexpected. It suggests that B-cell responses to a single lymphokine can differ depending on the stage in the cell cycle of the B cell and also that different costimulators of B cells can direct the cell toward different pathways of differentiation in the presence of a single lymphokine. The biology of few other cells is as complex. The molecular characterization of additional lymphokine regulators of B-cell responses will be required for the continued unraveling of that complexity.

We have made the surprising discovery that a neuronal growth factor is a lymphokine modulator of B-cell function. The factor, named neuroleukin, was initially purified from mouse salivary gland and a complementary DNA (cDNA) encoding the factor has been cloned (*6*). Recombinant neuroleukin supports the continued survival in culture of spinal and sensory neurons. Messenger RNA (mRNA) encoding neuroleukin is found in the brain and we hypothesize that neuroleukin may also be important for the growth and function of neurons within the central nervous system. We now report that neuroleukin is a lymphokine. Neuroleukin is a secretory product of lectin-stimulated T cells and induces immunoglobulin synthesis by cultured human peripheral blood mononuclear cells. We hypothesize that the B lymphocyte is the target of neuroleukin action in the Ig induction assay.

The amino acid sequence of neuroleukin is partially homologous to the sequence for the external envelope protein (gp120) of HTLV-III/LAV (*6*). The homology is to a segment of the *env* gene that is conserved in all HTLV-III/LAV viral isolates for which sequence information has been reported (*7*). HTLV-III/LAV is the causative agent of the acquired immune deficiency syndrome (AIDS) (*8*); however, its known cytopathy for T4 lymphocytes is not thought sufficient to be the sole cause of the immune deficiency observed in AIDS patients (*9*). HTLV-III/LAV is now known to infect the brain and to be associated with a subacute encephalitis and progressive dementia of unknown etiology (*10*). The partial homology of the external envelope protein of HTLV-III/LAV and neuroleukin, a growth factor that affects the function of both neurons and lymphocytes, may be important for understanding the pathology of the AIDS virus for both the brain and the immune system.

Production of neuroleukin by lectin-stimulated T cells. T cells can be induced to secrete lymphokines in vitro by stimulation with plant lectins. Treatment with concanavalin A (Con A) or phytohemagglutinin (PHA), for example, induces T cells to secrete IL-2 as well as other partially characterized factors (*11*). We find that such lectins also induce the secretion of neuroleukin by T cells.

Synthesis and secretion of neuroleukin by cultured cells was assayed by metabolic labeling with [^{35}S]methionine. Human peripheral blood mononuclear cells (MNC's) were obtained from healthy donors and stimulated in vitro by culture with Con A, PHA, or pokeweed mitogen (PWM) (*12*). After 3 days in culture, cells were incubated with [^{35}S]methionine, and the labeled protein secreted into the medium was collected (*13*). Secretion of ^{35}S-labeled neuroleukin was determined by specific immunoprecipitation with a polyclonal rabbit antiserum to rat neuroleukin and analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*14*).

Mononuclear cells freshly isolated from peripheral blood did not synthesize and secrete detectable neuroleukin (Fig. 1). Neuroleukin production was induced by any one of the three lectins tested—Con A, PHA, or PWM. Because we obtained neuroleukin production with all three lectins, we thought it is likely that neuroleukin was a T-cell product, since all three mitogens stimulate T-cell proliferation and lymphokine production. To determine whether neuroleukin was a T-cell, B-cell, or monocyte (macrophage) product, we stimulated MNC's with PWM for 3 days in culture and then fractionated them into cell subsets (*15*). A T-cell subset was obtained by rosetting with neuraminidase-treated sheep red blood cells (NSRBC's). After a second rosetting with NSRBC's, we obtained a B-cell subset from the nonrosetted cells by removing adherent cells through plating on tissue-culture plastic. The adherent cells represent the monocyte (macrophage) subset. T cells and B cells were identified by surface binding of the monoclonal antibody markers Leu-4 (T cells) and Leu-12 (B cells). Monocytes were identified by binding of Leu-M3. The T-cell subset that we obtained was routinely more than 96 percent Leu-4–positive, and the B-cell subset was routinely more than 95 percent Leu-12–positive, and less than 2 percent Leu-4–positive. In the B-cell subset fewer than 1 to 3 percent of the remaining cells were monocytes. In each experiment, the PWM-stimulated T-cell subset produced neuroleukin, whereas the B-cell

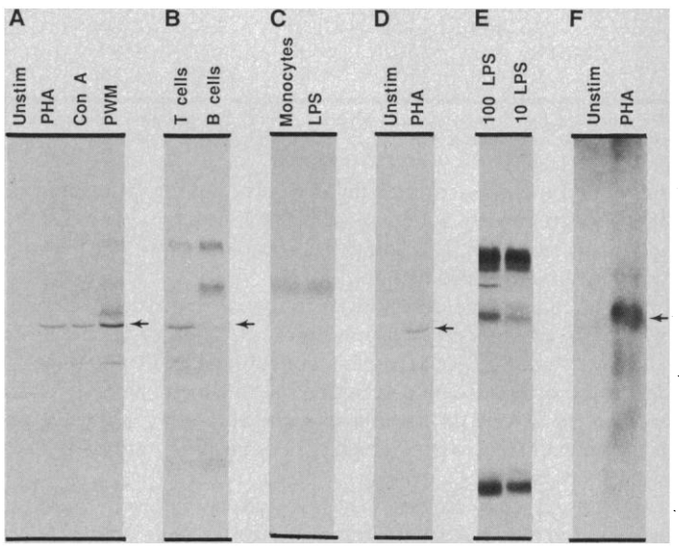

Fig. 1. Neuroleukin is a lectin-stimulated T-cell product in both mouse and man. In experiments A to E, lymphoid or myeloid cells were incubated with [^{35}S]methionine, and production of radiolabeled neuroleukin was detected by specific immunoprecipitation with rabbit antibody to rat neuroleukin. The immunoprecipitates were analyzed on reducing 10 percent SDS-polyacrylamide gels, and radiolabeled proteins were detected by autoradiography. Control immunoprecipitations with the preimmunization serum were negative. Mouse neuroleukin has a slightly greater electrophoretic mobility than human neuroleukin in the gel system used. The difference in mobility is seen more clearly in Fig. 4. (A) Human peripheral blood mononuclear cells were cultured for 3 days alone (Unstim), or with one of three lectins (PHA, Con A, or PWM), and then were incubated with [^{35}S]methionine. Each of the lectins induces production of neuroleukin (arrow). (B) Human MNC's were stimulated with PWM for 3 days in vitro, separated into T-cell and B-cell subsets, and then incubated with [^{35}S]methionine. Neuroleukin is specifically a T-cell product. (C) Human monocytes isolated from peripheral blood were cultured for 12 hours alone (monocytes) or were treated for 1 hour with LPS at 10 μg/ml and then were incubated with [^{35}S]methionine. No production of neuroleukin was detected. (D) Mouse spleen cells were cultured for 72 hours alone (Unstim) or with PHA; a T-cell subset was prepared by depletion of B cells and adherent cells and then incubated with [^{35}S]methionine. PHA-stimulated mouse T cells produce neuroleukin. (E) Mouse splenic B cells were depleted of T cells by treatment with antibody to Thy-1.2 and complement, were polyclonally activated to Ig secretion by culture for 3 days with LPS at 100 μg/ml or 10 μg/ml and then were incubated with [^{35}S]methionine. No production of neuroleukin was detected. In (F), expression of neuroleukin mRNA was determined by Northern blotting. Mouse spleen cells were stimulated with PHA, a T-cell subset was prepared, and RNA was extracted from the cells with a guanidine-isothiocyante procedure (*6*). Total cellular RNA from stimulated (PHA) and unstimulated (Unstim) T cells (25 μg per lane) was fractionated on a denaturing Formalin-agarose gel, transferred to nitrocellulose, and then probed for mRNA encoding neuroleukin with a single-stranded ^{32}P-labeled probe prepared from the mouse neuroleukin cDNA (*6*). Neuroleukin mRNA (~2 kb) was detected in PHA-stimulated T cells (small arrow) which was absent from unstimulated cells.

Table 1. Suppression of PWM-stimulated Ig secretion by monoclonal antibodies to neuroleukin. All cultures except the unstimulated control were treated with PWM. B109.9 and B216 are rat mAb's to neuroleukin. B109.9 binds to neuroleukin and inhibits the neurotrophic activity of the factor. B216 also binds to neuroleukin but is not inhibitory. The mAb's were prepared in ascites fluid and were added to the MNC cultures at dilutions of 1:80 or 1:400. Peripheral blood MNC's were obtained from three different human donors (KN, TR, and KC). The Ig concentration was determined in the culture supernatant 7 days after stimulation with PWM.

Treatment	Ig concentration (ng/ml)				Percent inhibition
	KN	TR	KC	Mean	
Unstimulated	140	380	80	200	
Addition					
PWM	3500	3100	2950	3180	
+B109.9 (1:80)	250*	820*	1950*	1010*	68
(1:400)	1600	5500	2500	3200	
+B216 (1:80)	3650	6600	3800	4680	
(1:400)	2500	4200	5100	3930	

*Significant suppression occurred with each donor; the percent inhibition of the average PWM response is given.

subset and the adherent cells failed to produce the factor (Fig. 1). We also stimulated adherence-selected monocytes with LPS to induce secretion of IL-1 and other monokines (*16*), yet failed to detect neuroleukin production.

We extended these observations to mouse in order to determine whether T cell–independent polyclonal activation of splenic B cells (*17*) could induce B-cell synthesis of neuroleukin. Mouse splenic B cells were activated to Ig secretion by treatment with LPS after removal of T cells by treatment with antibody to Thy-1.2 and complement (*18*). No production of neuroleukin by activated B cells was observed (Fig. 1).

To ensure that mouse T cells could be induced to secrete neuroleukin, we harvested inguinal, periaortic, and popliteal lymph nodes from mice and stimulated the cells by culture with PHA for 3 days. We also obtained splenic T cells by depleting B cells and monocytes through adherence to petri dishes coated with goat antibody to mouse Ig (*18*). Phytohemagglutinin-stimulated mouse T cells produced neuroleukin, whereas unstimulated T cells did not (Fig. 1). Thus, in mouse, as well as in man, neuroleukin is specifically a T-cell product and its production is induced in vitro by lectin stimulation.

Control of neuroleukin expression occurs at the level of mRNA. The availability of a mouse neuroleukin cDNA enabled us to probe Northern blots of total cellular RNA obtained from PHA-stimulated or unstimulated mouse T cells. Neuroleukin mRNA was detected in T cells only after stimulation with PHA (Fig. 1).

Recombinant neuroleukin induces polyclonal Ig secretion. A mouse neuroleukin cDNA has been inserted into an expression vector and transiently expressed by transfection into monkey COS-1 cells (*6*). Recombinant neuroleukin was obtained in a serum-free culture supernatant (designated C19) from the transfected cells. As a control, a serum-free culture supernatant (designated Mock) was collected from COS-1 cells that were transfected with the expression vector alone.

When added to cultured human peripheral blood MNC's, recombinant neuroleukin induces maturation of B cells into antibody-secreting cells. B-cell secretion of antibody was determined by a quantitative enzyme-linked immunosorbent assay (ELISA), and antibody-secreting cells were enumerated by a protein A–hemolytic plaque assay (*19*). A B-cell response to neuroleukin was demonstrated in both assays. For comparison, the human peripheral blood MNC's were also stimulated with PWM. PWM is the only plant lectin known to induce polyclonal B-cell maturation to Ig secretion (*20*). Recombinant neuroleukin (C19) and PWM each elicit comparable stimulation of Ig secretion (Fig. 2). In each case, the response measured is about ten times the baseline response. In parallel assays in which we measured Ig secretion and enumerated plaque-forming cells (PFC), we found both indices of B-cell function increased coordinately. For example, in one experiment the optimal dilution of C19 elicited Ig at 9000 ng/ml (unstimulated control, 580 ng/ml) assayed with ELISA and 1670 PFC (unstimulated control, 360 PFC) with the plaque-forming assay. In the same experiment, PWM elicited Ig at 7200 ng/ml and 2050 PFC.

Pokeweed mitogen evokes the differentiation to antibody-secreting cells of a subset of B lymphocytes that express IgG on their surface (*21*). To investigate whether neuroleukin and PWM might be evoking differentiation of different subsets of B cells, we examined the possibility that the response to costimulation with the two agents was additive. Mononuclear cells from three different individuals were used in the experiment; however, in each case, PWM, C19, or PWM plus C19 all elicited equivalent production of Ig (*22*). Thus, it seems unlikely that PWM and neuroleukin evoke differentiation of distinct B-cell subsets.

A difference between the in vitro responses elicited by neuroleukin and PWM is that PWM induces an early phase of cellular

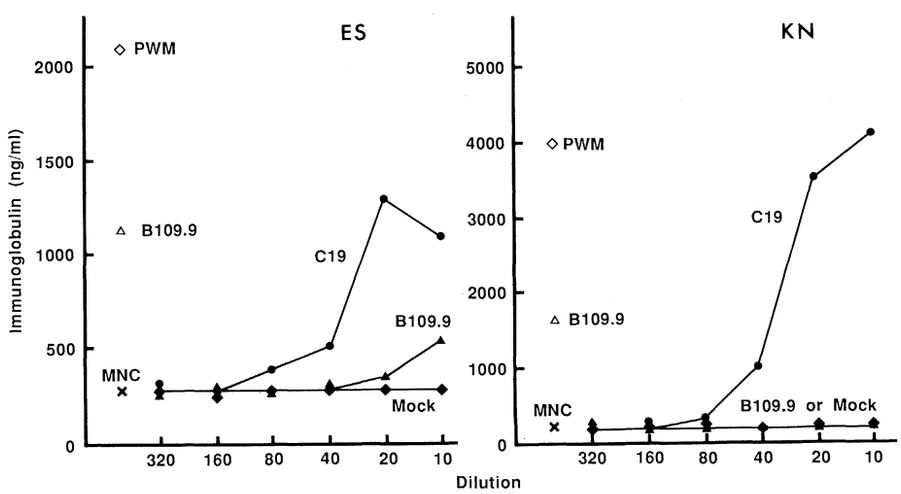

Fig. 2. Recombinant neuroleukin induces Ig secretion by cultured human MNC's. Results obtained with two different human donors (ES and KN) are illustrated. Immunoglobulin content was assayed by ELISA after 7 days of culture. Recombinant neuroleukin (designated C19) was obtained as a serum-free supernatant from transfected COS-1 cells that were transiently expressing mouse neuroleukin. A control (designed Mock) was provided by serum-free culture supernatant that was collected from COS-1 cells that were transfected with the expression vector alone. Immunoreactive neuroleukin is detected in C19 but not in Mock (*6*). C19 (●) contains an Ig-inducing factor that is absent from Mock (◆). Monoclonal antibody to neuroleukin when added together with C19 (C19 + B109.9) at a 1:80 dilution (▲) blocks the Ig-inducing activity of C19. Thus, the Ig-inducing factor in C19 is recombinant neuroleukin. For comparison, the response to PWM and its inhibition by mAb B109.9 is also shown (x) unstimulated MNC's; (◇) MNC + PWM; (△) MNC + PWM + B109.9 at a 1:80 dilution.

Table 2. Suppression by monoclonal antibodies and by antisera to neuroleukin of the activity of an Ig-inducing factor produced endogenously by cultured mononuclear cells after pokeweed mitogen stimulation. All cultures except the unstimulated control were treated with PWM. The Ig concentration was determined in the culture supernatant 7 days after stimulation with PWM. B109.9 is an inhibitory rat mAb to neuroleukin (an IgM). 774 is a rat mAb (an IgM) that reacts with a cell-surface determinant on myelin and macrophages. Pre and immune designate the preimmune and immune sera obtained from a rabbit immunized with purified rat neuroleukin. KN, MD, and DT refer to the MNC donors.

Treatment	Ig concentration (ng/ml)							
	In 10 percent FBS				In serum-free medium			
	KN	MD	DT	Percent inhibition	KN	MD	DT	Percent inhibition
Unstimulated	50	50	50		125	50	150	
Addition								
PWM	3500	2150	2400		2600	2100	1500	
+ B109.9 (1:80)	1900*	1200*	600*	54	800*	900*	800*	60
+ 774 (1:80)	3500	1900	1300		2850	2300	1800	
+ Pre (1:100)	3100	1800	2000		2700	1950	1800	
+ Immune (1:100)	2100*	1150*	1500*	41	1200*	1500*	900*	42
+ Immune (1:200)	3100	1950	1800		3200	1900	1800	

*Significant suppression of the PWM response was obtained with each of the MNC donors, the percent inhibition of the mean PWM response for the three MNC donors is given.

proliferation that is missing in neuroleukin-treated cultures. In one experiment, for example, at 48 hours after stimulation, [^{3}H]thymidine incorporation in PWM-treated cultures was 9900 count/min (unstimulated control, 930 count/min), whereas [^{3}H]thymidine incorporation in neuroleukin-treated cultures was baseline (570 count/min). By 7 days, when Ig levels are peaking, [^{3}H]thymidine incorporation is equivalent in neuroleukin- and PWM-stimulated cultures (51,640 and 49,300 count/min, respectively; unstimulated control, 24,650 count/min; triplicate wells). We believe that the proliferation measured early after PWM stimulation is a T-cell mitogenic response to PWM that is absent from the response to neuroleukin.

The potency of recombinant neuroleukin in the Ig-induction assay described above is similar to that in the neuron survival assays described by Gurney *et al.* (*6*). Half-maximal Ig induction or half-maximal stimulation of cultured spinal or sensory neuron survival occurs with about the same amount of C19 added to each assay medium. In experiments in which parallel Ig induction and neuron survival assays were performed, the titers observed for C19 in the two assays (about a 1/40 or 1/80 dilution) were equivalent within the limits of experimental error. The potency of neuroleukin purified from a tissue source has been determined in the spinal neuron survival assay. Half-maximal increase in the survival of spinal neurons is obtained with 0.7 ng of neuroleukin per milliliter, which corresponds to a molar concentration of $1.25 \times 10^{-11} M$ in the assay medium. Thus, we estimate that recombinant neuroleukin is active at a similar concentration in the Ig induction assay.

Two experiments showed that recombinant neuroleukin in the C19-transfected cell supernatant is responsible for the induction of Ig secretion measured. First, the Mock serum-free culture supernatant, collected from COS-1 cells transfected with the expression vector alone, did not contain immunoreactive neuroleukin (*6*). When tested in the Ig induction assay, the Mock supernatant did not induce Ig secretion. Thus, transfection of COS-1 cells with an expression vector construct that contains the mouse neuroleukin cDNA specifically directs synthesis of an Ig-inducing factor. Second, monoclonal antibodies (mAb's) to neuroleukin block the effect of C19-transfected cell supernatant in the Ig induction assay. We used two different mAb's to neuroleukin in the experiment (*23*). The first mAb (designated B109.9) is a rat IgM that binds to neuroleukin and inhibits the biological activity of the factor as measured in the neuron survival assays (*6*). The second mAb (designated B216) is a rat IgG2b that also binds to neuroleukin, but the binding of B216 does not inhibit the neurotrophic activity of the factor. When B109.9 or B216 (prepared in ascites fluid) were each added together with C19 to the Ig-induction assay medium, B109.9 blocked the induction of Ig secretion by C19, whereas B216 was not inhibitory (Fig. 2). Thus, the Ig-inducing factor in C19 is recombinant neuroleukin.

Monoclonal antibody to neuroleukin suppresses PWM-stimulated Ig secretion. The results described above suggested that the triggering of polyclonal B-cell maturation to antibody-secreting cells in vitro by PWM might be neuroleukin-dependent. We tested the neuroleukin dependence of the response to PWM by including mAb to neuroleukin in the PWM assay medium. Initial experiments compared the effects of adding mAb B109.9 or mAb B216 in ascites fluid to the MNC culture medium. B109.9 consistently inhibited PWM-stimulated Ig secretion by MNC's obtained from several different human donors, whereas B216 was not inhibitory (Table 1). The degree of suppression in the presence of B109.9 varied among MNC donors and ranged from 95 percent suppression to 22 percent (29 ± 4 percent, mean ± SEM; 18 donors tested). The mitogenic response of cultured MNC's to PWM stimulation assessed at 3 days on the basis of [^{3}H]thymidine incorporation was not affected (*24*). Mitogenic responses of cultured MNC's to Con A were similarly unaffected by mAb to neuroleukin.

Since the two mAb's to neuroleukin are of different subclasses, a concern was that the suppression of PWM-stimulated Ig secretion by B109.9 might be a nonspecific effect due to inclusion of a rat IgM in the assay medium. However, a collection of rat IgM mAb's to irrelevant antigens did not inhibit PWM-stimulated Ig secretion (*25*). Thus, the inhibition of PWM-stimulated Ig secretion by B109.9 was specific to the antibody.

We also tested the polyclonal rabbit antiserum to rat neuroleukin described in Fig. 1 for suppression of the PWM response. Consistent suppression of PWM-stimulated Ig secretion by MNC's from three representative donors was obtained with the antiserum to neuroleukin, whereas the serum obtained before immunization was not inhibitory (Table 2).

Finally, we wished to be certain that the inhibition of PWM-stimulated Ig production by the various immunochemical reagents was not due to inactivation of a serum component present in the assay medium. Mononuclear cells were stimulated with PWM during culture in a serum-free, defined medium (*26*). Once again, both B109.9 and rabbit antiserum to neuroleukin inhibited PWM-stimulated Ig production, whereas the appropriate control for each was without effect (Table 2).

Neuroleukin is produced by T cells within 3 days of culture with

PWM, but at 3 days little Ig has been synthesized (Table 3). To determine whether neuroleukin acts early or late in the in vitro response to PWM, we added the blocking mAb, B109.9, to the MNC assay medium at different times after exposure to PWM. Suppression of PWM-elicited Ig secretion was obtained only when the monoclonal antibody was added early (day 0) (Table 3); addition of B109.9 at day 3 or later did not inhibit subsequent Ig production. Thus, once B lymphoblasts have differentiated into antibody-secreting cells, Ig production is neuroleukin-independent.

Accessory cell requirement for neuroleukin-dependent Ig induction. Since neuroleukin induces Ig production by cultured human MNC's, we hypothesized that neuroleukin acts directly on B lymphocytes. However, our attempts to show this directly by removal of monocytes or T cells or through the use of various B-lymphocyte costimulators have so far been negative.

Neuroleukin-dependent Ig induction is both monocyte- (macrophage) and T cell–dependent. Mononuclear cells typically contain 10 to 20 percent monocytes; removal of monocytes by adherence to plastic leaves 1 to 3 percent monocytes as assayed by surface binding of Leu-M3. This degree of monocyte depletion significantly reduced Ig production in response to both PWM and neuroleukin and suggests that monocytes are required. To investigate further the apparent monocyte dependence of the neuroleukin response, we treated MNC's with the lysosomotropic agent L-leucine methyl ester (LME), a treatment that results in loss of monocyte function and viability (*27*). Treatment of MNC's with LME abrogated the neuroleukin response, and addition of low numbers (1 to 3 percent) of adherence-purified monocytes to LME-treated MNC's completely restored the response to neuroleukin (Fig. 3). Thus, LME-sensitive, plastic-adherent monocytes are required for neuroleukin-induced Ig production.

Removal of T cells also abrogated neuroleukin-dependent Ig production by the remaining MNC's. Highly purified human peripheral blood B cells were prepared by removing T cells by rosetting with NSRBC's and removing monocytes by adherence to plastic. The resultant B-cell subset responded to the B-cell mitogen *Staphylococcus aureus* Cowan I, but did not differentiate into Ig-secreting cells when cultured with neuroleukin. This finding suggests an accessory cell requirement. Further experiments in which adherence-purified monocytes were added back to B cells that were depleted of both T cells and monocytes did not restore neuroleukin responsiveness, an indication that T cells are required.

Neuroleukin is not BCGF-II, HMW-BCGF, or BCDF. Few soluble lymphokines are known that act directly on resting B lymphocytes. The induction of Ia expression by treatment of quiescent mouse splenic B cells with IL-4 is one of few B-cell (or T-cell) responses that can be elicited in the absence of accessory cells (*3, 28*). Accessory cell requirements have been eliminated in some in vitro assays of B-cell immune responses by use of a variety of costimulators. For example, cross-linking of cell surface IgM by a specific antibody to the mu chain renders B lymphocytes competent to respond to a number of different mitogenic factors (*2, 29, 30*). Other in vitro assays utilize costimulation with dextran sulfate (*31, 32*), or protein A insolubilized in the cell wall of Formalin-fixed *S. aureus* (SAC) (*33, 34*).

Table 3. Neuroleukin is an early acting factor required for PWM stimulation of Ig secretion. KN and ES are different MNC donors. PWM was added to all of the cultures on day 0. B109.9 was added at a final dilution of 1:80 on the day indicated. The Ig concentration was determined in the culture supernatant by ELISA. N.D., not determined.

Day	Ig concentration (ng/ml) on day indicated	
	KN	ES
0	0	0
3	630	530
5	2100	1000
7	3900	2550
After addition of B109.9 on day indicated (Ig content assayed on day 7)		
0	1650*	1150*
3	4000	2100
5	4200	N.D.

*Significant inhibition.

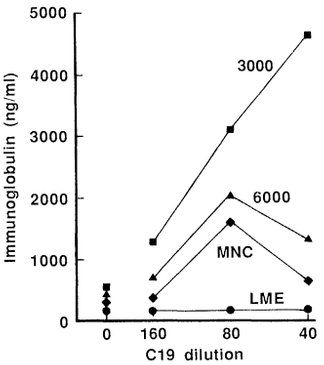

Fig. 3. Monocyte dependence of Ig induction by neuroleukin. Human MNC's were treated with 5 m*M* L-leucine methyl ester (LME) for 40 minutes at room temperature to deplete monocytes. The cells were then washed and cultured (2×10^5 per 0.2 ml) with the indicated dilutions of recombinant neuroleukin in C19. Untreated human MNC's respond to neuroleukin with induction of Ig synthesis (◆), whereas monocyte depletion by LME treatment abrogates the response (●). Addition of 3000 (■) or 6000 (▲) adherence-purified monocytes back to LME-treated, monocyte-depleted MNC's restored responsiveness to neuroleukin.

Neuroleukin, a 56-kD polypeptide, is an unusually large factor. Most other polypeptide factors including the interleukins, epidermal and fibroblast growth factors, nerve growth factor or platelet-derived growth factor, and the colony-stimulating factors have molecular sizes in the range of 10 to 30 kD. Thus, several partially characterized B-cell growth factors (BCGF's) or B-cell differentiation factors (BCDF's) were of particular interest as their reported molecular sizes are between 50 and 60 kD (*29, 31, 32*). The first of these, BCGF-II, induces proliferation of mouse splenic B cells in an assay that utilizes costimulation with dextran sulfate (*31*). The second, high molecular weight BCGF (HMW-BCGF), induces proliferation of human tonsilar B cells in an assay that utilizes costimulation with antibody to human Ig (*29*).

Neuroleukin preparations, known to have bioactivity by Ig induction, were tested in BCGF-II and HMW-BCGF assays at doubling dilutions that bracketed the optimal Ig-inducing concentration. A relevant positive control in the BCGF-II assay was provided by a cell-free supernatant harvested from PHA-stimulated human MNC's at 48 hours (*32*). A similar supernatant harvested from PWM-stimulated MNC's at 72 hours provided a positive control in the HMW-BCGF assay. Several preparations of neuroleukin tested in either BCGF assay did not stimulate proliferation of purified B lymphocytes (Table 4). Neuroleukin also had no detectable effect on the proliferation of human tonsilar B cells costimulated with SAC (Table 4). B lymphocytes stimulated with SAC respond by enhanced proliferation to IL-2 and possibly other factors (*33*). In addition, neuroleukin did not directly induce detectable proliferation of either human or murine purified B cells or unseparated lymphocyte preparations. Thus, neuroleukin had no detectable BCGF activity.

The ability of neuroleukin to induce the differentiation or maturation of B-cell tumor lines was also determined. The B-lymphoblastoid cell line CESS (*34*) is induced to secrete IgG by T cell–derived differentiation factors (*35*) while the pre-B mouse tumor cell line 70Z/3 responds to certain B-cell maturation factors (BMF's) by expressing surface immunoglobulin (sIg) (*36*). This cell line has

been demonstrated to respond to both IL-1 and IFN-γ (*37*). Neuroleukin had no detectable effect on IgG secretion by CESS cells or on the expression of sIg by 70Z/3 cells and thus, has no BCDF or BMF activity in these assays (Table 4). Since IL-1 can also affect B-cell differentiation (*38*), any potential IL-1 activity of neuroleukin was assessed in the mouse thymocyte comitogenesis assay (*39*). Neuroleukin had no detectable IL-1 activity in this assay.

Expression of neuroleukin in bone marrow and by human cell lines of lymphoma-leukemia origin. An initially puzzling observation that led to the studies described above is that neuroleukin is expressed at high levels in bone marrow. We surveyed expression of neuroleukin in various tissues by Western blotting as described for mouse (*6*). We extended the study to include rabbit and selected human tissues. Skeletal muscle, brain, and bone marrow were the tissues that consistently expressed the highest levels of neuroleukin in the three species studied (Fig. 4). Mouse is unusual in that varying amounts of neuroleukin were detected in all tissues examined; we did not detect neuroleukin in other rabbit or human tissues examined.

We also examined the production of neuroleukin by established cell lines. Neuroleukin was not produced by the adherent cell lines examined, including mouse fibroblasts (3T3), Chinese hamster ovary (CHO) cells, and monkey kidney (COS-1) cells. However, all human cell lines of lymphoma-leukemia tumor origin that we examined produced detectable neuroleukin (Fig. 4). These included tumor cell lines derived from T- and B-lymphoblastoid and myeloid (granulocyte-macrophage) lineages (*40*). Thus, neoplastic transformation of B lymphoblasts or myeloblasts allows neuroleukin expression by cells that normally do not produce the factor.

We hypothesized that transformation of blast cells within the lineages examined induces expression of neuroleukin. This could be tested in the B-cell lineage by transformation with Epstein-Barr virus (EBV). Infection with EBV polyclonally activates B lymphocytes to Ig secretion and can be used to derive Ig-secreting lymphoblastoid cell lines that are immortalized to growth in culture (*41*). We found that infection of purified human B cells with EBV (*42*) induces neuroleukin expression with a time course that parallels induction of logarithmic cell proliferation and polyclonal activation of Ig synthesis (Fig. 4). Lymphoblastoid cell lines that were derived from virus-infected cultures have continued to express neuroleukin indefinitely.

Early after EBV infection of a human B-cell culture, "bystander" activation of uninfected B lymphocytes to antibody-secreting cells is observed (*43*). We hypothesize that this may be due in part to neuroleukin secretion by EBV-infected cells. To determine whether continued secretion of Ig by an EBV-positive, lymphoblastoid cell line was dependent on production of neuroleukin by these cells, we cultured the cells in the presence of B109.9. However, no diminution of Ig production was observed. Thus, an early event in the activation of Ig production by EBV-infected lymphocytes may be neuroleukin-dependent. However, continued production of Ig by EBV-positive lymphoblastoid cell lines is neuroleukin-independent, as also was demonstrated for PWM-induced antibody-secreting cells.

Role of neuroleukin in the B-cell immune response. We detected the lymphokine activity of neuroleukin in a simple Ig-induction assay with human peripheral blood MNC's, although the cell-cell interactions and soluble lymphokines produced endogenously in the culture, which act in concert with neuroleukin, are complex. Not unexpectedly, the characterization of the neuroleukin target cell in the Ig-induction assay, as well as accessory cell and costimulating factor requirements, has proved difficult. We know that neuroleukin acts early in assays that measure Ig induction in vitro. That was shown directly by adding an inhibitory mAb to neuroleukin to MNC cultures that had been stimulated with PWM. We also believe that neuroleukin acts early in the neuroleukin-dependent Ig induction assay, since the biological activity of neuroleukin preparations deteriorates rapidly in culture media or on storage. We hypothesize that an early event that triggers activation or clonal expansion of B lymphocytes is neuroleukin-dependent. Production of Ig by mature antibody-secreting cells, however, is neuroleukin-independent. Late addition of an inhibitory mAb to neuroleukin to PWM-stimulated cultures, at a time when substantial numbers of antibody-secreting cells have been induced, does not turn off Ig secretion. We also found that autocrine stimulation by neuroleukin-producing EBV-positive, B-lymphoblastoid lines was not required for continued Ig secretion.

A difference between PWM-stimulated and neuroleukin-stimulat-

Table 4. Activity of recombinant neuroleukin in lymphokine assays. Recombinant neuroleukin was obtained in a serum-free supernatant (C19) collected from transfected COS-1 cells that were transiently expressing mouse neuroleukin. A range of dilutions of C19 that bracketed the optimal Ig-inducing dilution determined for each batch were used in the assays. Assays were repeated several times with at least two different batches of C19. The BCGF assay with antibody to human Ig as costimulator (*31*) was performed as follows. Isolated tonsil B cells (5×10^4 per 0.2 ml) were costimulated with goat $F(ab')_2$ fragment of antibody to human IgM (8.5 μg/ml) (TAGO) and C19 for 72 hours. During the last 18 hours of culture, the cells were incubated with [^{3}H]thymidine (6 Ci/mmol, 1 μCi per well), and then incorporated label was determined. The negative control was treated with antibody to human Ig alone. The positive control was a supernatant from PWM-stimulated human MNC's collected at 72 hours. For the BCGF assay with *S. aureus* as costimulator (*35*), isolated tonsil B cells (5×10^4 per 0.2 ml) were costimulated with 0.0025 percent Formalin-fixed *S. aureus* (Calbiochem) and C19 for 72 hours. [^{3}H]thymidine incorporation was determined as above. The negative control was treated with *S. aureus* alone. The positive control was recombinant IL-2 (Cetus). For the BCGF-II assay with dextran sulfate as costimulator (*33*), isolated BALB/c mouse splenic B cells (5×10^4 per 0.2 ml) were costimulated with dextran sulfate (25 μg/ml) and C19 for 72 hours. [^{3}H]thymidine incorporation was determined as above. The negative control was treated with dextran sulfate alone. The positive control was a supernatant from PHA-stimulated human MNC harvested at 48 hours. For the IL-1 assay (*41*), mouse thymocytes (5×10^5 per 0.2 ml) were costimulated with 2 percent PHA and C19 for 72 hours. [^{3}H]thymidine incorporation was determined as above. The negative control was treated with PHA alone. The positive control was a supernatant from LPS-pulsed (1 hour at 37°C) human MNC's harvested at 48 hours. For the BCDF assay (*37*), CESS cells (American Type Culture Collection) were cultured (1×10^4 per 0.2 ml) with C19 for 72 hours. Secreted Ig was determined by ELISA. The negative controls were untreated CESS cells. The positive control was a supernatant from PWM-stimulated human MNC's harvested at 72 hours. For the BMF assay (*38*), 70Z/3.12 cells (American Type Culture Collection) were cultured (10^5 per milliliter) with C19 for 48 hours. Surface Ig-positive (sIg$^+$) cells were identified by fluorescence microscopy after reaction with rhodamine-labeled rabbit antibody to mouse Ig. The negative controls were untreated 70Z/3.12 cells. The positive control was a supernatant from LPS or Con A–stimulated mouse spleen cells harvested at 72 hours.

Target cell	Negative control	Positive control	Neuroleukin
	BCGF assay with antibody to Ig (count/min)		
Tonsil B lymphocytes	100	1,950	220
	BCGF assay with S. aureus *(count/min)*		
Tonsil B lymphocytes	9,315	24,460	8,750
	BCGF-II assay with dextran sulfate (count/min)		
Mouse B splenocytes	78	2,890	60
	IL-1 assay (count/min)		
Mouse thymocytes	1,340	17,995	1,250
	BCDF assay (Ig in ng/ml)		
CESS cells	190	640	225
	BMF assay (percent sIg$^+$)		
70Z/3.12 cells	3	95	3

ed Ig induction is that PWM elicits an early T-cell proliferative response, whereas no early cell proliferation is seen after treatment with neuroleukin. However, both PWM- and neuroleukin-treated cultures exhibit considerable cell proliferation late in the assays, at a time when Ig levels have peaked. Thus, neuroleukin, unlike PWM, does not evoke a direct mitogenic response, although clonal expansion of B lymphocytes prior to transformation into antibody-secreting cells is a component of the response to neuroleukin.

We found no evidence that neuroleukin is a mitogen. Neuroleukin was not mitogenic for purified human or mouse B cells, it lacked B-cell growth factor activity in well-defined assays that measure B-cell proliferation in response to costimulation with antibody to immunoglobulin or dextran sulfate (*31–34*), nor did a blocking monoclonal antibody to neuroleukin inhibit mitogenic responses to PWM stimulation by cultured human MNC's. In addition, neuroleukin did not induce differentiation of proliferating B lymphoblasts into antibody-secreting cells in assays that have been used to identify BCDF's (*36, 37*). B-cell differentiation factors are envisioned to act late in the B-cell immune response to trigger transformation of a B lymphoblast that has undergone clonal expansion into a population of antibody-secreting plasma cells (*1*). Our evidence indicates that neuroleukin acts much earlier in the in vitro B-cell immune response.

We tend toward the view that neuroleukin acts on B lymphocytes. However, neuroleukin may be an autocrine factor that acts on the T cell itself or a T-cell product that amplifies monocyte function. Either action would be one that is required early for induction of B lymphocyte responses in vitro. The next step in the characterization of the biology of neuroleukin will be to identify the cellular receptor for the factor. Of interest will be to determine which lymphoid and neuronal cell populations express neuroleukin receptors, whether the neuronal and lymphoid receptors are the same or different, and whether expression of the receptor on lymphoid cells is cell cycle– or lineage-dependent.

As described in the preceding article (*6*), the amino acid sequence of neuroleukin is partially homologous to a segment of the gp120 gene product of HTLV-III/LAV which is highly conserved in all virus isolates that have been sequenced (*7*). A number of clinical studies indicate that ongoing polyclonal activation of B lymphocytes occurs in AIDS patients. Humoral immunity is severely impaired (*44*), yet serum antibody levels are increased (*44, 45*). B lymphocytes isolated from peripheral blood have been polyclonally activated in vivo (*45*), and increased numbers of B blast cells are observed in lymph nodes of AIDS patients in association with retroviral particles (*46*). Detergent-lysed HTLV-III/LAV preparations also are reported to have a direct effect on human B-cell immune responses in vitro (*47*). We hypothesize that such findings indicate that the partial sequence homology of gp120 to neuroleukin enables it to mimic the biological activity of neuroleukin. If so, this suggests a common mechanism whereby an HTLV-III/LAV gene product may be both immunosuppressive and interfere with neuronal function.

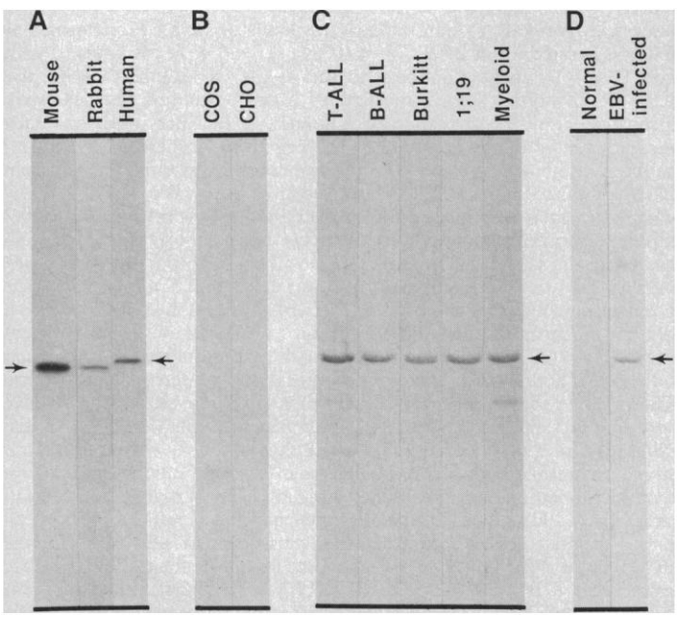

Fig. 4. Neuroleukin is present in bone marrow and is produced by human cell lines of lymphoma-leukemia origin. In experiment A, neuroleukin was detected in tissue homogenates of bone marrow from mouse, rabbit, and man by Western blotting (*6*). When compared to the mouse or rabbit factor, human neuroleukin is of slower electrophoretic mobility in the gel system used. In experiments B to D, production of neuroleukin by cultured cell lines was determined by radioimmunoprecipitation as in Fig. 1. (B) The adherent cell lines examined, which included monkey kidney COS-1 cells, Chinese hamster ovary (CHO) cells, mouse L-cell fibroblasts, and mouse 3T3 cell fibroblasts did not produce detectable neuroleukin. All cell lines of human lymphoma-leukemia origin (*40*) examined produced neuroleukin. These included acute lymphoblastic leukemia (T-ALL) cell lines of T-cell origin (MOLT 4 illustrated, and SUP T1), acute lymphoblastic leukemia (B-ALL) cell lines of early B-cell origin (SUP B13, and RS4;11 illustrated), Burkitt lymphoma (Burkitt) cells (Jijoye illustrated, Raji, and Daudi), cells established in culture from EBV-negative, B-cell lymphomas including Loukes (c-*myc* translocation) and chromosome 1;19 translocations (1;19; 697 and RCH-ACV illustrated), and also myeloid leukemia cell lines (myeloid; HL60 illustrated, and ML2). (D) Infection with Epstein-Barr virus induced expression of neuroleukin by transformed human B cells. Freshly isolated human B cells (normal) do not produce detectable neuroleukin. A T cell–depleted human MNC culture was infected with EBV in the presence of cyclosporin. Seven days after EBV infection, the culture initiated logarithmic proliferation, and polyclonal secretion of Ig was induced. At that time, production of neuroleukin by the cultured cells was first detected. Lymphoblastoid cell lines derived from the infected culture continued to produce neuroleukin indefinitely.

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12. Mononuclear cells were isolated on a Ficoll-Hypaque gradient from freshly drawn peripheral venous blood donated by adults (age, less than 50 years). Cells were suspended in Hanks balanced salt solution, washed ten times at 4°C, and suspended in culture medium at 10^6 cells per milliliter. Except for the studies with serum-free medium, the medium used was RPMI 1640 supplemented with 10 percent fetal bovine serum (FBS), 4 nmol of glutamine, 20 m*M* (*N*-morpholino)propanesulfonic acid, and gentamycin (0.1 ng/liter). For assay of lectin-stimulated proliferation, microwell cultures were labeled at 3 days with 0.5 μCi of [^{3}H]thymidine (6 Ci/mmol, Amersham) for 5 hours.
13. Microwell MNC cultures were labeled for 6 hours by incubation with [^{35}S]methionine (200 μCi/ml, 13 Ci/mmol) in 100 μl of methionine-free RPMI.
14. Immunoprecipitations were performed in 0.01*M* tris-HCl (*p*H 7.2), 0.15*M* NaCl, 1 percent Triton X-100, 0.1 percent sodium dodecyl sulfate, 5 m*M* EDTA, and ovalbumin at 2 mg/ml. The supernatant was first precipitated by reaction with insoluble protein A (Formalin-fixed *S. aureus*; Immunoprecipitin, Bethesda Research Laboratories), and then incubated overnight with 2 μl of rabbit antibody to

rat neuroleukin (*6*). Rabbit IgG with bound antigen was then precipitated with insoluble protein A and analyzed by SDS-PAGE and autoradiography.
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18. B cells were obtained from the spleens of BALB/c mice. The spleens were mechanically disrupted and washed; red blood cells were lysed by hypotonic shock, T cells were lysed with antibody to Thy-1.2 (IgM) and rabbit complement (adsorbed with mouse cells). Removal of T cells as described abrogated the mitogenic response to PHA. To obtain T cells, B cells and monocytes were removed as described by L. J. Wysocki and V. L. Sato [*Proc. Natl. Acad. Sci. U.S.A.* **75**, 2844 (1978)]. The T-cell subset contained fewer than 3 to 4 percent surface Ig-positive cells as assessed by immunofluorescent staining with antibody to mouse IgG. Mouse spleen cells were stimulated with PHA (1:100) in medium containing 5 percent FBS for 72 hours in vitro. T cell–depleted, splenic B cells were activated to Ig secretion by culture for 3 days with LPS at 10 to 100 μg/ml.
19. For assay of neuroleukin- or PWM-stimulated Ig induction, MNC's were obtained from donors known to be high responders to PWM (more than 1000 ng of Ig per milliliter). A 0.2-ml portion of the MNC cell suspension was cultured with PWM or dilutions of C19 in round-bottom 96-well plates (Costar) for 7 days at 37°C in a humidified incubator at 5 percent CO_2. Supernatants of quadruplicate cultures were pooled and assayed by ELISA for Ig content with antibodies to human IgG (H + L) and biotinylated goat antibody to human Ig (Vectastain, Vector Labs), as previously described [M. Rosenkoetter, A. T. Reder, J. J-F. Oger, J. P. Antel, *J. Immunol.* **132**, 1779 (1984)]. Enumeration of antibody-secreting cells by a protein A–hemolytic plaque assay was performed as described [R. R. Bernabe *et al.*, in *Immunological Methods*, I. Lefkovits and B. Pernis, Eds. (Academic Press, New York, 1982), vol. 2, p. 187].
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24. Results obtained with PWM were as follows (five MNC donors): PWM alone, 44,740 ± 5,910 (counts per minute mean ± SEM); PWM + B109.9 (1:80), 43,500 ± 5,060 count/min.
25. Five different rat IgM monoclonal antibodies prepared against the torpedo electroplaque acetylcholine receptor [D. P. Richman *et al.*, *Nature (London)* **286**, 738 (1980)] were tested. The amounts of Ig obtained were as follows: with PWM alone, 5100 ng/ml; with PWM plus rat IgM mAb's, 5590 ± 810 ng/ml (mean ± SD for five mAb's). A rat IgM (designated 774) reactive with a cell-surface determinant on myelin and macrophages [K. Stefannson, A. T. Reder, J. P. Antel, *J. Neuroimmunol.* **12**, 49 (1986)] was also used in some experiments.
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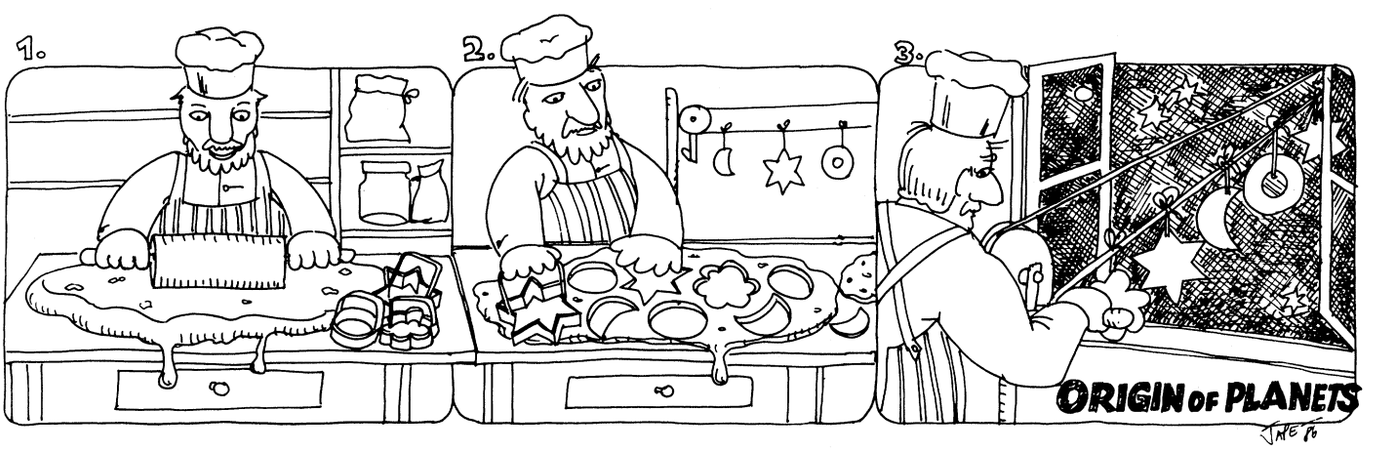

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