Brief Definitive Report

GENERATION OF A LYMPHOCYTE GROWTH FACTOR BY TREATMENT OF HUMAN CELLS WITH NEURAMINIDASE AND GALACTOSE OXIDASE*

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The oxidizing mitogens, sodium periodate and galactose oxidase, induce proliferation of T cells by generation of aldehyde moieties on cell surface glycoproteins (1–3). A brief (30-min) incubation of lymphocytes with these agents is sufficient to generate the mitogenic signal. The oxidizing agent can then easily be removed by washing the cells. These procedures provide a useful tool for investigation of soluble factors that are released from activated lymphocytes and that may play a role in mediating lymphocyte activation. Blastogenic factors have been reported in the supernates of cells treated with mitogenic lectins (concanavalin A [Con A] [4, 5] or phytohemagglutinin [PHA] [6, 7]) or with allogeneic lymphocytes (in mixed lymphocyte cultures [MLC] [8, 9]). These factors have a variety of biological activities, the most striking being the induction of proliferation in activated lymphocytes, the activation of cytotoxic T cells (CTL), and the maintenance of such cells in long-term tissue culture. We report here that a mitogenic or growth factor is produced by human lymphocytes activated by a brief treatment of the cells with neuraminidase and galactose oxidase (NAGO). The soluble factors produced by NAGO-stimulated lymphocytes are mitogenic for nonproliferating cells that have previously been exposed to mitogens or allogeneic cells, or that have been incubated without mitogens for 7–14 d. The NAGO-induced factors do not produce proliferation in freshly isolated, unprimed peripheral blood lymphocytes. Moreover, these soluble factors induce differentiation of MLC memory cells to secondary CTL.

Materials and Methods

Human peripheral blood mononuclear cells (PBL) were obtained from healthy normal volunteers, age 21–47 yr, by Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) -Hypaque (Winthrop Laboratories, New York) gradient centrifugation as previously described (10). Lectin-induced memory cells were prepared by adding PHA (2 μg/ml) or Con A (2 μg/ml) to PBL (10^6/ml) that were suspended in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) that contained 5% fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (RPMI-1640 medium). NAGO-induced memory cells were prepared by exposing PBL ($10–20 \times 10^6$/ml), suspended in phosphate-buffered saline (PBS), to 50 U/ml of neuraminidase and 2.6 U/ml of galactose oxidase for 30 min at 37°C in a shaking water bath. The treated PBL were then washed twice with PBS and resuspended in RPMI-1640 medium that contained D-galactose (5 mg/ml). The mitogen-treated memory cells

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were collected after 7–14 d of incubation at 37°C in a 95% air – 5% CO_2 humidified atmosphere. Long-term primary MLC cells (MLC memory cells) were prepared as previously described (11). Soluble factors were prepared as follows: Cells were treated with either NAGO (NAGO-primed medium) or no mitogen (unprimed medium), as described above, and medium was collected by centrifugation after varying times (24–48 h). D-Galactose (5 mg/ml) was added to the media that were then, in some cases, filtered through millipore filters (0.2-μm pore size) and frozen until use. The mitogenic effects of the soluble factors were assayed on both freshly isolated PBL and on the various memory cells. The target cells (2×10^6 cells/ml) were distributed (100 μl/well) in microtiter plates. 100 μl of either fresh medium or the various soluble factors was then added. After varying time periods, [^{3}H]thymidine incorporation (2 μCi/well) was determined over a 16-h interval. Mitogen responsiveness of the various target cells was also assessed by direct stimulation with Con A (2 μg/ml) or PHA (2 μg/ml). To assess the generation of secondary CTL, MLC memory cells were cocultured for an additional 4 d with equal numbers of syngeneic cells (responder cells in the primary MLC) or allogeneic cells (stimulator cells in the primary MLC) along with either 50% fresh medium or 50% of the soluble factors. At the end of the culture period the cells were harvested, washed, and used as effector cells in a 4-h ^{51}Cr-release assay (11). Specificity of secondary cytotoxic activity was established by using the original sensitizing cells as the specific target.

Results

Effects of NAGO-primed Medium on Mitogen-induced Memory Cells. The effect of NAGO-primed medium on stimulating [^{3}H]thymidine incorporation into either freshly isolated cells or mitogen-induced memory cells was investigated. Mitogen-induced memory cells were prepared by treating PBL with NAGO and incubating them at 37°C for 7–14 d. The peak blastogenic response to NAGO occurs in 2–4 d, and by 7 d there is minimal residual incorporation of [^{3}H]thymidine (1,500 ± 520 cpm/culture). Fig. 1 illustrates the results of these experiments. The effect of 50% fresh medium, unprimed medium, or NAGO-primed medium on [^{3}H]thymidine incorporation into three types of cells, fresh cells, cells incubated 7–14 d without mitogen, and mitogen-induced memory cells was determined. Neither fresh medium or unprimed medium induced significant blastogenesis in any of the cell types. NAGO-primed medium, on the other hand, induced a marked blastogenic response in mitogen-induced memory cells (17,800 ± 1,700 cpm/culture), a moderate response in preincubated cells (8,200 ± 2,010 cpm/culture), and a minimal response in freshly prepared cells (2,664 ± 273 cpm/culture). [^{3}H]Thymidine incorporation was also determined between 0 and 16, 24 and 40, and 48 and 64 h in cultures of freshly isolated cells and NAGO-induced memory cells after treatment with PHA directly (2 μg/ml), 50% NAGO-primed medium, or 50% unprimed medium (Fig. 2). The mitogen-induced memory cells respond more rapidly to PHA than do freshly prepared cells. The NAGO-primed medium is more effective in inducing blastogenesis in mitogen-induced memory cells than in freshly isolated cells at each time interval tested. Resting cells, 7–14 d after stimulation with PHA (2 μg/ml) or Con A (2 μg/ml), also proliferated in response to NAGO-primed medium (data not shown). Fresh and unprimed medium are again seen to be ineffective in activating memory cells (Fig. 2). To determine the kinetics of the production of the activating factor, NAGO-primed medium was harvested 24 and 48 h after treatment of the cells with NAGO. In addition, medium was changed after 24 h, and the 24- to 48-h NAGO-primed medium was also tested. [^{3}H]Thymidine incorporation was stimulated in mitogen-induced memory cells by 0- to 24 (19,600 ± 980 cpm/culture) and 0- to 48-h (18,200 ± 625 cpm/culture) NAGO-primed medium, but not by 24- to 48-h NAGO-primed medium (3,200 ± 710 cpm/culture).

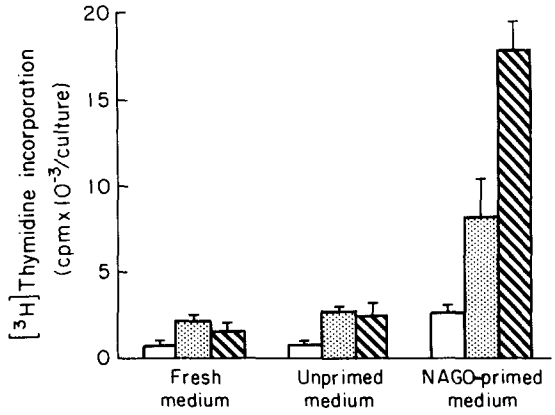

FIG. 1. Effect of supplementation of culture medium with 50% fresh medium, unprimed medium, or NAGO-primed medium on the proliferative response ([^{3}H]thymidine incorporation, cpm × 10^{-3}/culture) of PBL (□) PBL preincubated for 7–10 d (preincubated cells) (▨) and NAGO-induced memory cells (▧). Results of five experiments ± SEM.

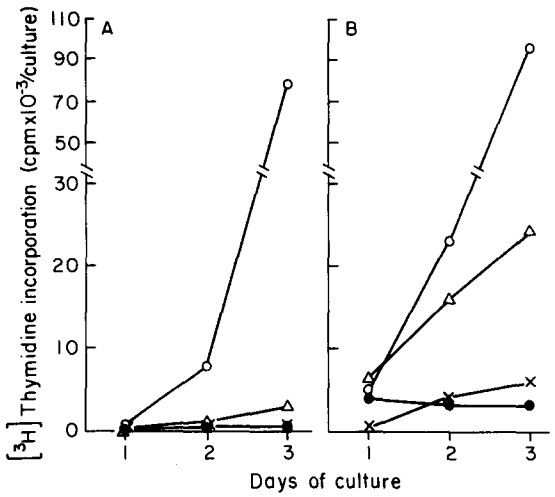

FIG. 2. Effect of direct stimulation of (A) PBL or (B) NAGO-induced memory cells with PHA (2 μg/ml) (○) and of supplementation of culture medium with 50% fresh medium (●), unprimed medium (×), or NAGO-primed medium (△) on [^{3}H]thymidine incorporation. Results are those of a typical experiment.

NAGO-primed medium prepared in the absence of serum was as effective as that prepared in the presence of 5% fetal calf serum (data not shown).

Effect of NAGO-primed Medium on MLC-induced Memory Cells. Because NAGO-primed medium activates resting cells after mitogen stimulation but does not activate freshly prepared cells, we wondered if immunologically specific memory cells would be similarly activated. The effect of NAGO-primed medium on long-term primary MLC cells (MLC memory cells) generated in 11 allogeneic combinations is shown in Table I. The memory cells were harvested 2–3 wk after initiation of the primary MLC, washed, resuspended in either unprimed or NAGO-primed medium, and cocultured with irradiated syngeneic cells for an additional 4 d. The unprimed and primed

TABLE I

*Activation of MLC Memory Cells By NAGO-primed Medium and Allogeneic Cells**

	Syngeneic cells	P‡	Allogeneic cells	Unprimed medium	P‡	NAGO-primed medium
Proliferation, *cpm*§	1,896 ± 338	<0.001	12,318 ± 1,208	1,396 ± 230	<0.001	12,735 ± 2,097
Cytotoxicity, *% SCR*‖	16.9 ± 6.9	<0.02	42.9 ± 12.1	13.6 ± 5.6	<0.02	39.1 ± 9.5

* Results of 11 experiments ± SEM.

‡ *P* value determined by paired *t* test.

§ Proliferation was assessed by [^{3}H]thymidine incorporation (2 μCi/well) 72–86 h after incubation of the MLC memory cells with irradiated syngeneic or allogeneic cells or with unprimed or NAGO-primed medium in addition to irradiated syngeneic cells.

‖ Percent SCR = ER − SR/MR − SR × 100, where ER = counts in experimental group, SR = spontaneously released counts, and MR = counts released by incubating targets in distilled water.

media were obtained from cells of the individuals whose cells functioned as responder cells in the primary MLC. Thus, the possibility of alloantigen in the NAGO-primed medium was eliminated. Separate aliquots of memory cells were also suspended in fresh medium and cocultured with irradiated syngeneic cells to assess residual or baseline activity or with irradiated allogeneic cells (original stimulating cells in the primary MLC). NAGO-primed medium induced significant proliferation in the MLC-induced memory cells, as assessed by [^{3}H]thymidine incorporation, between 48 and 64 h after initiation of the cultures. Unprimed and fresh medium did not induce proliferation. The extent of proliferation of MLC memory cells cultured in the presence of NAGO-primed medium (12,735 ± 2,097 cpm/culture) was essentially the same as that occurring in MLC memory cells cocultured with the irradiated original stimulating cells in the primary MLC (12,318 ± 1,208 cpm/culture).

In addition to induction of proliferation, we also found that NAGO-primed medium induced differentiation of MLC memory cells to specific secondary CTL. For these experiments, PBL from individuals whose cells were the stimulator cells in the primary MLC were the specific targets and cells from individuals whose cells functioned as responder cells in the primary MLC were used as syngeneic targets. Specific ^{51}Cr release (SCR) was 16.9 ± 6.9 and 13.6 ± 5.6%, respectively, in MLC memory cells resuspended in fresh or unprimed medium (residual cytotoxicity). NAGO-primed medium induced a significant increase in cytotoxicity (39.1 ± 9.5% SCR) that was essentially the same as that induced by the original stimulating cells (42.9 ± 12.1% SCR) (Table I). Cytotoxicity was not evident when syngeneic cells were used as targets.

Discussion

This study demonstrates that NAGO treatment of human PBL results in the generation of a growth factor that is mitogenic for nonproliferating cells that have previously been exposed to mitogen or alloantigen. Cells incubated for 7–14 d in the absence of mitogen or alloantigen are also responsive to the growth factor. The oxidizing mitogens are unique in that they induce lymphocyte activation by introduction of aldehyde groups on cell-surface glycoproteins. Only a brief exposure of the cells to the mitogenic oxidizing agents is sufficient to generate the mitogenic signal, and, after this, the oxidizing agent can be completely removed from the system. Thus, supernates that contain factors produced by activated lymphocytes may be obtained

in the total absence of mitogen or antigen. Despite washing the cells after NAGO treatment, residual galactose oxidase might have been carried over to the NAGO-primed medium. We therefore added D-galactose to the NAGO-primed medium to eliminate galactose oxidase activity on cells by substrate competition. Preliminary studies indicate that medium from $NaIO_4$-activated lymphocytes has mitogenic properties similar to the NAGO-primed medium. The mitogenic oxidizing agents thus provide a useful tool for the production of soluble factors that may be assayed before purification to remove residual mitogen or antigen.

The target for the NAGO-induced growth factor appears to be generated by either a polyclonal or antigenic (MLC) stimulus. Our finding that preincubated cells also proliferate in response to NAGO-primed medium suggests that either preincubation itself or exposure to serum is sufficient to generate receptors for the mitogenic factor. Increased reactivity of T cells to a lymphocyte-derived mitogenic factor after rosetting of T cells has previously been reported (12). Activation of secondary CTL can be achieved by reexposure to alloantigen (13, 14) or by stimulation with polyclonal T cell mitogens (15–17), including NAGO (18, 19), or with soluble factors produced during the course of an MLC (20), or after Con A stimulation (21). Our study indicates that the NAGO-primed medium is effective in stimulating secondary CTL in the absence of residual mitogen or antigen. For these studies the NAGO factor was prepared from cells syngeneic to the responding cells in the MLC, thus eliminating the possibility of alloantigen shed into the medium. The ability to generate a potent lymphocyte growth factor in the absence of lectin, alloantigen, or serum provides a useful system for purification and characterization of this material. Molecular properties of the NAGO-generated growth factor and its relation to other lymphocyte-generated growth factors are currently under investigation.

Summary

Supernates of neuraminidase and galactose oxidase (NAGO)-treated lymphocytes induce blastogenesis in nonproliferating cells harvested 7–14 d after treatment with mitogen or alloantigen and in cells incubated without mitogen for 7–14 d but not in freshly isolated peripheral blood lymphocytes. Virtually all the growth factor is produced by NAGO-treated cells during the first 24 h of incubation, and no increase in factor activity is detected upon further cell culture. Serum is not required for growth factor production. NAGO-primed medium induces generation of specific cytotoxic T cells from mixed lymphocyte culture (MLC) memory cells to approximately the same extent as that induced by allogeneic cells (stimulating cells in the primary MLC). NAGO-primed medium provides a useful reagent for isolation and characterization of lymphocyte growth factors and other lymphokines.

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