Chemical reduction of oxidized human lymphocytes inhibits interleukin 2 production but not induction of interleukin 2 responsiveness
(neuraminidase plus galactose oxidase/sodium borohydride/anti-Tac monoclonal antibody/human thymocytes)

EHUD ROFFMAN*, BENJAMIN SREDNI†, ARAM SMOLINSKY‡, AND MEIR WILCHEK*
*Department of Biophysics, Weizmann Institute of Science, Rehovot, Israel; †Department of Life Sciences, Bar-Ilan University, Ramat Gan, Israel; and ‡Department of Cardiac Surgery, Chaim Sheba Medical Center, Tel Hashomer, Israel
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ABSTRACT Treatment with neuraminidase (NA) plus galactose oxidase (GalOxase) does not cause stimulation of human thymocytes. However, stimulation can be achieved by addition of exogenous interleukin 2 (IL-2). The IL-2-induced stimulation was inhibited with anti-Tac antibody, indicating that NA/GalOxase-oxidized cells can serve as inducers of functional IL-2 receptors on IL-2-responding T cells. The induction of IL-2 receptors by the oxidized cells was not inhibited by subsequent reduction with borohydride, since the cells could still be stimulated with IL-2. The presence of IL-2 receptors was also confirmed by flow cytometry using indirect immunofluorescence. Peripheral blood lymphocytes can be stimulated by NA/GalOxase treatment, and the conditioned medium from this treatment can support the growth of an IL-2-dependent line. This stimulation can be inhibited with borohydride and restored with IL-2. The conditioned medium derived from the borohydride-reduced cells cannot support the growth of the IL-2-dependent line, indicating that borohydride inhibits the oxidation-induced IL-2 production. The results suggest that NA/GalOxase-oxidized sites can be modified chemically without losing the potential to induce IL-2 receptors.

Resting lymphocytes can be activated by mitogens and antigens and by chemical or enzymatic oxidation. The activation has been considered to be dependent on crosslinking of the mitogen receptors on the cell surface membrane, resulting in the induction of DNA synthesis and subsequent mitosis (1).

Studies with lectins indicate that mitogenic activation of lymphocytes is at least a two-step process (2). Binding of the mitogen to the cell surface causes the simultaneous production of interleukin 2 (IL-2) by one set of cells (IL-2 producers) and induction of IL-2 receptors in another population of cells (IL-2 responders).

Oxidative mitogens, another group of polyclonal T-cell stimulators (3), also appear to exert their blastogenic effects through interleukins (4, 5). It has been shown that oxidation-induced mitogenesis is inhibited by aldehyde-blocking agents such as sodium borohydride (6, 7). Therefore, it was of interest to examine which stage(s) of the process is inhibited by borohydride: production of IL-2 or induction of IL-2 receptors. In the present study, it was found that borohydride inhibits IL-2 production without affecting induction of IL-2 responsiveness, since full stimulation can be restored by addition of primate IL-2.

Anti-Tac monoclonal antibody has recently been identified as a putative antibody against the IL-2 receptor in human T cells (8) and has been shown to block many IL-2-dependent T-cell-mediated immune reactions (9). We therefore decided to investigate whether oxidative mitogenesis is also associated with Tac antigen expression.

Here we provide evidence that incubation of human T lymphocytes with neuraminidase (NA) plus galactose oxidase (GalOxase) results in the expression of Tac antigen and concomitant induction of IL-2 responsiveness. Moreover, after the reduction step, anti-Tac binding potential was retained, confirming that functional IL-2 receptors are expressed on the reduced lymphocytes. Such studies on functionally isolated cell populations may shed additional light on the mechanism of T lymphocyte activation.

MATERIALS AND METHODS

Materials. Phytohemagglutinin (PHA) and affinity-purified goat anti-mouse IgG labeled with fluorescein isothiocyanate were obtained from Bio-Yeda (Rehovoth, Israel); Vibrio comma NA was from Behringwerke and Dactilium dendroides GalOxase was from Sigma. To inactivate residual enzyme contaminants, GalOxase was dissolved to 100 units/ml in phosphate-buffered saline (pH 7.3; P/NaCl) and incubated for 30 min at 50°C. NaBH₄ was obtained from Fluka. [methyl-3H]Thymidine (5 Ci/mmol, 1 Ci/ml; 1 Ci = 37 GBq) was from Nuclear Research Center (Negev, Israel). The ascites fluid containing monoclonal anti-Tac antibody was provided by T. A. Waldmann (National Cancer Institute, Bethesda, MD). Peanut agglutinin (PNA) was obtained from departmental sources.

Preparation of MLA144 Conditioned Medium. The MLA144 cell line was provided by H. Rabin from the LBI-Basic Research Program (NCI-Frederick Cancer Research Facility, Frederick, MD). Serum-free conditioned medium from the Gibbon T-cell line MLA144 was prepared according to Rabin et al. (10). This line releases IL-2 constitutively, without detectable levels of other lymphokines and cytokines (11). Briefly, MLA144 cells were grown in 200-ml tissue culture flasks (Falcon 3024) in RPMI 1640 medium containing fetal bovine serum. When the cell density reached 2 × 10⁶/ml, the cells were washed three times with RPMI 1640 medium, reseeded in serum-free medium at 2 × 10⁶/ml and incubated at 37°C for 48 hr. The cells were then removed by slow-speed centrifugation and the conditioned medium was filtered through a 0.45-µm filter unit (Falcon 7102). This filtrate will be referred to below as SF-MLA144-CM.

Cell Preparation. A portion of fresh normal thymus was obtained from patients, aged 7 mo to 4 yr, following corrective heart surgery (more than 20 samples were studied). Sep-

Abbreviations: NA, neuraminidase; GalOxase, galactose oxidase; PNA, peanut agglutinin; IL-2, interleukin 2; SF-MLA144-CM, serum-free conditioned medium from the MLA144 cell line; PBL, peripheral blood mononuclear leukocytes; P/NaCl, phosphate-buffered saline (pH 7.3); PHA, phytohemagglutinin.
aration of thymocyte subpopulations with PNA to PNA– (mainly mature) and PNA+ (mainly immature) cells was carried out according to Reisner et al. (12). Peripheral blood mononuclear leukocytes (PBL) were separated by Ficoll-Hypaque centrifugation from the blood of healthy donors.

**Oxidation and Reduction.** For NA/GalOxase oxidation, the cells were suspended in P/NaCl (2 x 10⁷ cells per ml) and gently shaken at 37°C for 30 min with NA at 0.04 unit/ml and GalOxase at 2.5 units/ml and then extensively washed with P/NaCl containing d-galactose at 5 mg/ml to remove excess enzyme and inhibit residual GalOxase activity.

For borohydride reduction, cells were suspended in cold P/NaCl (2 x 10⁷ cells per ml), and a freshly prepared solution of NaBH₄, was added to the cell suspension to a final concentration of 5 mM. The cells were incubated at 37°C for 30 min with occasional shaking and then washed three times with P/NaCl. Cell viability after both oxidation and reduction exceeded 95%, as determined by trypan blue exclusion.

**Microcultures.** Cells were cultured in a final volume of 200 µl (2 x 10⁷ cells per microwell) in microtiter plates (Costar 3596) in RPMI 1640 medium supplemented with 10% heat-inactivated pooled human serum A, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin at 200 units/ml, streptomycin at 200 µg/ml, and neomycin at 10 µg/ml. Where indicated, anti-Tac ascites, SF-MLA144-CM, or PHA was directly added to the plates. The cultures were incubated at 37°C in 5% CO₂/95% air for 72 hr. Ten hours before termination of culture, each well was pulsed with 1 µCi of [³H]thymidine. The cells were harvested and the radioactivity was determined in a liquid scintillation counter.

**Immunofluorescence Studies.** To detect Tac antigen by indirect immunofluorescence, cells were incubated in tissue culture dishes (Falcon 3003; 2 x 10⁷ cells per 20 ml) under the culture conditions described above. At different times, 5 x 10⁶ cells were washed with P/NaCl and then incubated for 30 min at 4°C in 0.6 ml of P/NaCl containing bovine serum albumin at 1 mg/ml and a 1:120 dilution of anti-Tac ascites. After two washes with P/NaCl, the cells were incubated under the same conditions with a 1:32 dilution of fluorescein-conjugated goat anti-mouse IgG. After two P/NaCl washes, the staining pattern was analyzed with a fluorescence-activated cell sorter (FACS II, Becton Dickinson). In each sample, 2 x 10⁵ cells were analyzed.

**IL-2 Assay.** To generate IL-2-containing supernatants, bulk cultures were prepared as described above. At 24 hr, the supernatants were collected, filtered through 0.22-µm Millipore filter units, and assayed for IL-2 activity according to Gillis et al. (13). Briefly, 10⁶ CTLL-2 (a cytotoxic IL-2-dependent T-cell line) cells per well were cultured in complete RPMI 1640 medium as described above, except that 5% fetal bovine serum was used instead of human serum and 50 µM 2-mercaptoethanol was included. After addition of supernatants, the culture was incubated for 24 hr and then pulsed with [³H]thymidine for another 16 hr. The culture was harvested and radioactivity was determined.

**RESULTS**

**Effect of NA/GalOxase on IL-2 Responsiveness in Human Thymocytes.** Treatment of unseparated or PNA– thymocytes with NA/GalOxase does not result in proliferation and thymidine uptake. On addition of IL-2, the thymocytes become strongly responsive. Neither native, NA-treated (data not shown), nor GalOxase-treated thymocytes responds to exogenous IL-2 (Fig. 1). These findings indicate that NA/GalOxase treatment of thymocytes does not result in IL-2 production but effectively induces IL-2 responsiveness. This was further confirmed by the use of anti-Tac monoclonal antibody, which is believed to bind to the IL-2 receptor on responder T cells. Anti-Tac ascites at a dilution of 1:1000.

**Effect of Borohydride on NA/GalOxase-Induced IL-2 Responsiveness.** Sodium borohydride inhibits the stimulation of lymphocytes induced by oxidative mitogens. As shown in Fig. 2, reduction with borohydride of PNA– thymocytes immediately after their oxidation with NA/GalOxase did not affect their ability to respond to IL-2. Borohydride reduction of nonoxidized cells had no effect on the induction of IL-2 responsiveness. In thymocytes from young patients, NA/GalOxase was not mitogenic. This may be due to the fact that such thymocyte preparations are nearly devoid of monocytes (14), an essential cellular component for the production of interleukin 1, which is a prerequisite for IL-2 production (2). However PBL, which do include accessory cells, can be activated by NA/GalOxase treatment and are inhibited by borohydride. The stimulation can then be restored with exogenous IL-2 (Fig. 3).

**Effect of Reduction on NA/GalOxase-Induced Tac Antigen Expression.** The ability of NA/GalOxase to induce Tac antigen expression was tested by flow cytometry using indirect immunofluorescence. The fluorescence distribution of the PNA– thymocytes shown in Fig. 2 is illustrated in Fig. 4A. After 16 hr of incubation, the fluorescence intensity exhibited by the sample that had been treated successively with NA/GalOxase and borohydride was less than that of the sample treated with NA/GalOxase alone. After 60 hr, however, the fluorescence distribution of the two samples superimposed. Cells not subjected to oxidation displayed only background fluorescence. The fluorescence pattern from unseparated PBL is shown in Fig. 4B. In this case, there was no significant difference in the behavior of the NA/GalOxase and NA/GalOxase–, NaBH₄-treated samples. Borohydride reduction of nonoxidized cells had no effect on Tac antigen expression (data not shown).
Effect of Borohydride on NA/GalOxase-Induced IL-2 Production by PBL. Our findings indicate that borohydride fails to affect IL-2 responsiveness but may affect IL-2 production. To examine this possibility, we used CTLL-2 cells (13) to study the proliferation induced by different conditioned media. It has been shown previously (4) that NA/GalOxase treatment of PBL results in IL-2 production. This has been confirmed in our studies (Table 1), which showed that borohydride treatment of NA/GalOxase-oxidized PBL inhibits the potential of these cells to produce IL-2, as indicated by the failure of CTLL-2 cells to proliferate in the presence of conditioned medium derived from these cultures. Cultures incubated with PHA were not affected by previous treatment with the reducing agent. In addition, borohydride reduction of nonoxidized cells had no effect on IL-2 production by native PBL.

DISCUSSION

The production of aldehyde groups on lymphocytes by mild oxidation with sodium periodate or with NA/GalOxase is an excellent tool to study the mechanism of lymphocyte activation (3, 5). The polyclonal lymphocyte activation induced by oxidation can be abolished by reduction with borohydride (6, 7). Oxidative mitogenesis can also be blocked by other aldehyde-specific groups such as hapten-containing hydrazides; the derivatized lymphocytes can then be activated with antibodies against the hapten (15). In the case of murine thymocytes, it was shown more than a decade ago that oxidation alone is not enough to cause stimulation and that accessory cells (or their products) are required (16).

In this study, we were able to clarify the growth factor requirements underlying the induction of oxidative mitogenesis in human thymocytes. T-cell activation in our system could be obtained by addition of IL-2, indicating that all the molecular components (with the exception of IL-2) required for stimulation were present on the cells by virtue of the oxidation procedure. This implies that oxidation of pure T cells does not result in IL-2 production but induces its putative activity.

Table 1. Effect of chemical reduction of oxidized PBL on their oxidation-induced IL-2 production

<table>
<thead>
<tr>
<th>CM added, %</th>
<th>[3H]Thymidine incorporation into CTLL-2 cells induced by various conditioned media, cpm</th>
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<tr>
<td>Native PBL</td>
<td>549</td>
</tr>
<tr>
<td>NaBH₄-treated PBL</td>
<td>767</td>
</tr>
<tr>
<td>NA/GalOxase-oxidized, NaBH₄-treated PBL</td>
<td>1222</td>
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PBL were subjected to the indicated treatments and cultured for 24 hr. To assay IL-2 activity, supernatants were collected and added to previously seeded CTLL-2 cells. Each culture was incubated for 40 hr and pulsed with [3H]thymidine 16 hr before termination. When no CM was added, the background value detected was 662 ± 89. PHA was used at 10 μg/ml. Results are expressed as arithmetic mean of quadruplicate wells; SD values were 5–15%.
membrane receptors, thereby causing stimulation. This observed stimulation could be inhibited by anti-Tac antigen, which is thought to recognize the human IL-2 receptor. Anti-Tac has been shown, in vitro, to block various human T-cell activation systems that are induced by antigens and mitogenic lectins (9). We have extended these observations to oxidative mitogens, thereby including oxidative mitogenesis in the same category.

It was essential to determine whether the persistence of aldehyde groups at the cell surface is required throughout the incubation period in order to induce IL-2 receptors. We therefore reduced the aldehyde groups with borohydride, and we found that the thymocytes respond equally well to IL-2. The presence of IL-2 receptors on the reduced thymocytes was also confirmed by indirect immunofluorescence.

To study which stage of the stimulation is inhibited by borohydride we used PBL, which, unlike thymocytes, can be stimulated by NA/GalOxase. Using this system, we have shown that borohydride inhibits IL-2 production. Thus, production of IL-2 and induction of its receptors are carried out by two different mechanisms. We have not yet determined whether the inhibition of IL-2 production is a direct result of borohydride-reduced T cells or whether the inhibition of IL-2 production by T cells is carried out by another mechanism—e.g., inhibition of interleukin 1 production by monocytes or induction of prostaglandin E2 synthesis (17). Nevertheless, at this stage it is clear that aldehyde groups must be present on the oxidized cells for IL-2 production but not for IL-2 receptor expression.

The observation of Larsson and Coutinho (18), using mitogenic amounts of PHA, which can selectively induce IL-2 receptors on mouse splenocytes, is somewhat similar to our results. However, the chemical nature of our system offers wider experimental possibilities for the molecular study of IL-2 receptor induction, because different aldehyde-blocking agents can be used to label the oxidized sites without the loss of IL-2 receptor expression. This system is also suitable for the study of cell–cell interactions, since signal transmission in oxidative mitogenesis can also be induced indirectly (5, 19).

We suggest that oxidized thymocytes contain cells that induce expression of the IL-2 receptor as well as cells which bear such receptors; however, unlike PBL, the thymocytes lack a functional IL-2 production system. We cannot yet exclude the possibility that the putative functions suggested for thymocytes may be carried out by the same T cell.

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