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# Effect of corticosteroids on the response of lymphocytes to stimulation by galactose oxidase-modified lymphocytes

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**Summary.** Human peripheral blood mononuclear cell preparations, after treatment by neuraminidase plus galactose oxidase, stimulated untreated lymphocytes. The increases in tritiated thymidine incorporation in the responder lymphocytes were observed after 48 h of mixed cell cultures. Monocyte-depleted lymphocyte preparations were equally effective stimulator cells. Both purified T and B fractions were effective stimulator cells. On the other hand, only the T but not the B fractions could respond to the stimulation. The response of the cells to this type of stimulation was suppressed by $10^{-4}$–$10^{-7}$ M of the corticosteroid preparation methylprednisolone. When the cells treated with neuraminidase plus galactose oxidase were cultured alone for 48 h, they lost their stimulating capacity. However this loss could not be prevented by the presence in the culture of methylprednisolone. Hence the drug has selective suppressive activity on one type of lymphocyte activity but not the other.

## INTRODUCTION

Although lymphocytes are classified into distinct subpopulations, these subpopulations can modify the activities of one another. Several *in vitro* models are available to examine such interaction in human lymphocytes. Among these, it has been found that lymphocyte preparations exposed to sodium periodate ($NaIO_4$) can stimulate untreated lymphocytes *in vitro* to increase their rates of DNA synthesis. In this model, the $NaIO_4$-treated lymphocytes are prevented from DNA synthesis by treatment with the reagent mitomycin C. The stimulator and responder lymphocytes can be from the same or different individuals. This reaction differs from that of the conventional mixed lymphocyte cultures in that the maximum increases in DNA synthesis are observed as early as 48 h after initiation of culture. In contrast, mixed lymphocyte cultures require 5–7 days to attain such increases (O'Brien, Parker, Paolilli & Steiner, 1974, Schmitt-Verhulst & Shearer, 1976).

The present authors obtained a similar reaction by treating the stimulator lymphocytes with neuraminidase plus galactose oxidase (NGAO) instead of $NaIO_4$. As with $NaIO_4$, the treated lymphocytes lost their ability to stimulate after being cultured alone for 48 h. Although the effect of corticosteroids on many different types of immunological reactions have been examined, their effect on this type of cellular interaction is still unknown (Nowell, 1961; Heilman, Gambrill & Leichner, 1973; Blomgren, 1974; Yu, Kacena & Pearson, 1976; Yu, 1976). We investigated the following questions:

(1) whether a monocyte-depleted preparation of lymphocytes can serve as stimulator cells. In the paper reported by O'Brien, no attempt was made to

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remove the monocytes (O'Brien *et al.*, 1974). Since $NaIO_4$ or NGAO treated monocytes can stimulate lymphocytes (Biniamov, Ramot & Novogrodsky, 1974; Biniamov *et al.*, 1975; Greineder & Rosenthal, 1975), it is important to know whether pure preparations of lymphocytes can still stimulate untreated lymphocytes. (2) Whether the stimulator lymphocytes are T or B subpopulations. (3) Whether the responder lymphocytes are T or B subpopulations. (4) The effect of corticosteroids on the mixed cell reaction. (5) The effect of corticosteroids on the stimulatory capacity of the treated cells.

## MATERIALS AND METHODS

Heparinized samples of peripheral blood were taken from normal human volunteers. Mononuclear cells were isolated by Ficoll–Hypaque gradient. Where indicated, monocytes were removed by incubation with iron powder as follows: The cells were suspended in RPMI-1640 with 10% heat inactivated foetal calf serum at $5 \times 10^6$ cells per ml and mixed with 40 mg per ml of carbonyl iron powder (GAF Corp., New York, U.S.A.). The tubes were incubated with constant rotation at 37° for 30 min. Afterwards, the iron powder was removed by a horseshoe magnet. These preparations contained less than 1% of monocytes as judged by intracellular peroxidase staining.

To treat cells with mitomycin, they were suspended at $5 \times 10^6$ per ml in Hanks's balanced salt solution (HBSS) with 50 $\mu$g/ml of mitomycin-C (Sigma, St. Louis, Missouri, U.S.A.). They were incubated for 30 min at 37° and washed three times prior to culture.

To treat cells with NGAO, they were washed twice with phosphate-buffered saline (PBS) and resuspended at $1–5 \times 10^7$ per ml in PBS with 0·5 u per ml of GAO (Worthington, Freehold, New Jersey, U.S.A.) and 50 u/ml of neuraminidase (Behring, Summerville, New Jersey, U.S.A.). The suspension was incubated at 37° for 30 min and washed once with HBSS. All culture media were purchased from Gibco, Grand Island Biological, New York, U.S.A.

Lymphocytes were cultured in 1 ml volumes of $1 \times 10^6$ per ml in $12 \times 75$-mm plastic culture tubes (Falcon # 2058) at 37° for 48 h. Two microcuries $\mu$Ci of tritiated thymidine ([$^3$H]TdR, 5 Ci/mmole, Amersham, Arlington Heights, Illinois, U.S.A.) were added for the last 4 h of culture. The cells were precipitated with 10% trichloroacetic acid and the amount of radioactivity incorporated assessed by scintillation-counting. Experiments were done in triplicate. Results were expressed as counts per minute.

To isolate lymphocyte subpopulations, monocyte-depleted lymphocyte preparations were suspended in HBSS at $5 \times 10^6$ cells per ml. Sheep red blood cells (SRBC) were obtained from Flow Lab, Rockville, Maryland, U.S.A. and kept at 4° in Alsevers solution. They were washed three times with isotonic saline and suspended at 1% concentration in HBSS with 10% heat-inactivated foetal calf sera. The latter were previously adsorbed with SRBC. 2·5 ml of lymphocyte suspensions and 2·5 ml of SRBC suspensions were mixed in $17 \times 100$-mm plastic tubes (Falcon # 2006), centrifuged at 200 *g* for 10 min and kept at 4° overnight. The pellets were gently resuspended. Two millilitres of Ficoll–Hypaque solution were gently injected with a syringe and Wintrobe needle under the rosette suspensions. The tubes were centrifuged at 400 *g* at 4° for 20 min. The interface and pellet cells were collected separately, washed once with HBSS and exposed to isotonic ammonium chloride solution at room temperature for 5 min. SRBC in the pellet samples were lysed by this solution. SRBC rosette-forming cells were assayed as described previously (Yu, 1976). The percentages of rosette-forming cells were $95·2 \pm 0·5$ (range 93–97) in the pellet samples and $5·4 \pm 1·4$ (range 1–9) in the interface. The percentage of SRBC rosette-forming cells before separation was $69·2 \pm 2·8$% (range 61–80). SRBC rosette-forming cells were designated as thymus derived (T) lymphocytes (Jondal, Holm & Wigzell, 1972). Non-rosetting cells were designated as bone marrow-derived (B) lymphocytes.

Methylprednisolone (Upjohn, Kalamazoo, Michigan, U.S.A.) was dissolved in HBSS. To test its effect, 0·1 ml of the solution was added to each ml of lymphocyte culture.

Results of experiments were calculated as averages $\pm$ standard errors of means. Statistical comparisons were made by paired observation *t*-test.

## RESULTS

### Cultures of NGAO-treated lymphocytes with untreated lymphocytes

Cells treated with NGAO and mitomycin were designated as stimulator cells (Ls). Untreated cells were designated as responder cells (Lr). The protocol reported by O'Brien *et al.* (1974) on mixed cell

**Table 1.** Radioactivity incorporated per culture by Lr, Ls and Lr plus Ls samples

| | Lr | Ls | Lr+Ls |
|---|---|---|---|
| Expt 1 | 700±100 | 4000±700 | 14,700±600 |
| Expt 2 | 500±50 | 9300±700 | 21,200±2200 |
| Expt 3 | 300±50 | 3100±300 | 7800±1400 |
| Expt 4 | 300±10 | 3000±300 | 14,100±400 |
| Expt 5 | 800±80 | 8000±400 | 17,700±1900 |
| Expt 6 | 2000±100 | 17,700±100 | 36,000±1000 |

Lr = untreated responder lymphocytes; Ls = mitomycin and NGAO-treated stimulator lymphocytes. Experiments were also done in which mitomycin and neuraminidase-treated cells were cultured with responder lymphocytes. No significant increases in [$^3$H]TdR incorporation were detected.

cultures with $NaIO_4$ treated lymphocytes was adopted. In each tube of mixed cell cultures, $0{\cdot}25 \times 10^6$ Ls were cultured with $1{\cdot}0 \times 10^6$ Lr in 1 ml of medium. Each tube of the control cultures consisted of $0{\cdot}25 \times 10^6$ Ls alone or $1{\cdot}0 \times 10^6$ Lr alone, also in 1 ml of medium. In any single experiment, the Ls and Lr might be from the same or two different individuals. The results of both combinations were similar and their results were analysed together. Cultures were carried out at 37° for 48 h. [$^3$H]TdR incorporation in the mixed cell cultures (Lr+Ls) was significantly higher than that in the Lr alone ($P<0{\cdot}005$) or Ls alone ($P<0{\cdot}001$) or the sum of Ls alone and Lr alone ($P<0{\cdot}001$) (Table 1).

**Effect of removal of monocytes from stimulator cells**

Monocytes were removed from the mononuclear cell preparations. The lymphocytes were then treated with mitomycin and NGAO and cultured with untreated mononuclear cell preparations. Significant increases in [$^3$H]TdR incorporation were still detected in the mixed cell samples compared to the sum of Ls alone samples and Lr alone samples ($P<0{\cdot}05$). There was no significant difference between the increases in the samples with and without monocytes ($P>0{\cdot}05$) (Table 2).

**Table 2.** Effect of removal of monocytes from stimulator cells

| | | Monocytes present in Ls | | Monocytes absent in Ls | |
|---|---|---|---|---|---|
| | Lr | Ls | Ls+Lr | Ls | Ls+Lr |
| Expt 1 | 900±100 | 8000±400 | 17,700±1900 | 2600±400 | 13,700±2700 |
| Expt 2 | 1100±100 | 12,600±400 | 31,900±1600 | 8600±100 | 22,500±1600 |
| Expt 3 | 300±10 | 3000±300 | 10,800±600 | 8000±500 | 13,000±300 |

Results expressed in c.p.m. of [$^3$H]TdR incorporated. Experiments were also done in which mitomycin and neuraminidase-treated cells were cultured with untreated cells. No significant increases in [$^3$H]TdR incorporation were detected.

**Table 3.** Effect of using purified T and B fractions as stimulator cells

| | | T fractions | | B fractions | |
|---|---|---|---|---|---|
| | Lr | Ls | Lr+Ls | Ls | Lr+Ls |
| Expt 1 | 1800±100 | 1300±100 | 24,100±700 | 400±40 | 30,500±1000 |
| Expt 2 | 1700±200 | 200±50 | 18,800±800 | 200±40 | 35,800±1300 |
| Expt 3 | 3000±300 | 300±30 | 10,400±600 | 800±100 | 22,200±500 |
| Expt 4 | 1900±300 | 600±40 | 12,400±400 | 100±20 | 6000±500 |

Results expressed in c.p.m. of [$^3$H]TdR incorporated. Ls+Lr samples were compared to the sums of Ls alone samples and Lr alone samples. This was $P<0{\cdot}02$ in the T fractions and $P<0{\cdot}05$ in the B fractions, $P>0{\cdot}05$ when the T fractions were compared with the B fractions.

Table 4. Effect of using T and B fractions as responder cells

| | | T fractions | | B fractions | |
|---|---|---|---|---|---|
| | Ls | Lr | Lr+Ls | Lr | Lr+Ls |
| Expt 1 | 10,100±700 | 1400±200 | 36,700±1700 | 700±300 | 9200±700 |
| Expt 2 | 23,800±2400 | 2400±200 | 35,400±1200 | 900±100 | 6700±600 |
| Expt 3 | 13,000±700 | 2000±100 | 36,400±300 | 1400±200 | 32,100±4500 |
| Expt 4 | 5200±500 | 1000±80 | 17,600±700 | 300±20 | 900±80 |

Results expressed in c.p.m. of [$^3$H]TdR incorporated. Ls+Lr samples were compared to the sums of Ls and Lr alone. This was $P<0{\cdot}02$ in the T fractions but $>0{\cdot}05$ in the B fractions.

**Effect of using purified preparations of T and B lymphocytes**

When Ls were prepared from isolated T and B lymphocytes and Lr from unseparated samples, both T and B fractions were able to induce increases in [$^3$H]TdR incorporation compared to the control samples (Table 3).

When Lr were prepared from isolated T and B lymphocytes and Ls from unseparated samples, only the T fractions could respond with increases in [$^3$H]TdR incorporation (Table 4).

**Effect of methylprednisolone on the mixed cell cultures**

Mixed cell cultures were carried out in the presence of $10^{-4}$–$10^{-8}$ methylprednisolone. In Tables 5 and 8, for clarity, only the increases in [$^3$H]TdR incorporation were shown. These were calculated by the formula:

c.p.m. in mixed cell sample −
(c.p.m. in Lr alone sample
+c.p.m. in Ls alone sample).

Significant suppression was observed with $10^{-4}$–$10^{-7}$ M of the drug ($P<0{\cdot}01$ for $10^{-7}$ M).

Table 5. Effect of methylprednisolone on Ls+Lr cultures

| Concentration of methylprednisolone (M) | Expt 1 | Expt 2 | Expt 3 | Expt 4 |
|---|---|---|---|---|
| 0 | 4400 | 16,300 | 11,800 | 6700 |
| $10^{-4}$ | 0 | 0 | 0 | 0 |
| $0{\cdot}5\times10^{-4}$ | 0 | 0 | 0 | 0 |
| $10^{-5}$ | 0 | 0 | 500 | 0 |
| $10^{-6}$ | 0 | 0 | 2400 | 0 |
| $10^{-7}$ | 1800 | 7200 | 4800 | 0 |
| $10^{-8}$ | 2900 | 6700 | n.d. | n.d. |

n.d. = Not done. Results expressed in c.p.m. of [$^3$H]TdR incorporated. Calculated by (Ls+Lr) samples minus (Ls samples alone plus Lr samples alone).

**Effect of culture on the stimulating capacity of the stimulator cells**

Lymphocytes were treated with NGAO and cultured for 48 h. They were then treated with mitomycin and used as stimulator cells. Responder cells were obtained from blood freshly drawn from the same

Table 6. Effect of 48 h culture on the stimulating activity of NGAO-treated cells

| | Before Culture | | | After Culture | | |
|---|---|---|---|---|---|---|
| | Ls | Lr | Ls+Lr | Ls | Lr | Ls+Lr |
| Expt 1 | 4000±700 | 700±100 | 14,700±600 | 200±100 | 300±10 | 300±10 |
| Expt 2 | 9300±700 | 500±30 | 21,200±2200 | 800±100 | 200±10 | 600±100 |

Results expressed in c.p.m. of radioactivity incorporated.

**Table 7.** Effect of methyl prednisolone on lymphocyte stimulation by NGAO

| | Expt 1 | Expt 2 | Expt 3 |
|---|---|---|---|
| Control | 2300 | 995±102 | 2102±145 |
| NGAO | 122,000 | 90,000 | 91,000 |
| NGAO+Methyl prednisolone $10^{-4}$M | 33,000 | 13,000 | 5000 |
| NGAO+Methyl prednisolone $5\times10^{-5}$M | 37,000 | 24,000 | 6000 |
| NGAO+Methyl prednisolone $10^{-5}$M | 58,000 | 37,000 | 12,000 |
| NGAO+Methyl prednisolone $10^{-6}$M | 77,000 | 49,000 | 23,000 |
| NGAO+Methyl prednisolone $10^{-7}$M | 128,000 | 65,000 | 47,000 |
| NGAO+Methyl prednisolone $10^{-8}$M | 109,000 | 78,000 | 68,000 |

Results expressed in c.p.m. of [$^3$H]TdR incorporated s.e. of each test < 20% of the average value.

**Table 8.** Effect of culture on the stimulating activity of Ls

| | Expt 1 | Expt 2 | Expt 3 |
|---|---|---|---|
| Before culture | 11,000 | 14,400 | 17,800 |
| After culture | −100 | 2000 | 1400 |
| Methyl prednisolone $10^{-4}$M | −400 | 2400 | 600 |
| Methyl prednisolone $10^{-5}$M | −100 | n.d. | 600 |
| Methyl prednisolone $10^{-6}$M | 600 | 1700 | 1200 |
| Methyl prednisolone $10^{-7}$M | 900 | n.d. | 1100 |
| Methyl prednisolone $10^{-8}$M | 500 | 1900 | 900 |

Results expressed in c.p.m. of [$^3$H]TdR incorporated. They are calculated by the formula: c.p.m. of Ls+Ls sample−(c.p.m. of Ls sample alone+c.p.m. of Lr sample alone). The column of methylprednisolone denotes that the Ls cells have been cultured with methylprednisolone for 48 h.

individual. The cells lost their stimulating ability after 48 hours of culture (Tables 6 and 8).

In several experiments, the NGAO treated lymphocytes were cultured for 48 h with $10^{-4}$–$10^{-7}$ M of methylprednisolone before culturing with Lr. These concentrations of methylprednisolone significantly inhibited the increased [$^3$H]TdR incorporation induced by the NGAO (Table 7). However, the stimulating capacity was still much diminished (Table 8).

## DISCUSSION

When lymphocytes are treated with $NaIO_4$, or NGAO, or even GAO alone, they become activated (Novogrodsky & Katchalski, 1972; 1973; Dixon, Parker & O'Brien, 1976). They develop increases in rates of DNA synthesis and become cytotoxic to some target cells (Schmitt-Verhulst & Shearer, 1976). The most likely reason is that these reagents induce the formation of aldehyde groups on the cell surfaces. These cross-link with neighbouring molecular groups to trigger the cells into activation. The increase in DNA synthesis can be inhibited by prior treatment of the cells with mitomycin, a DNA synthesis inhibitor. In the present study, the c.p.m. of [$^3$H]TdR incorporated were decreased from about 100,000 to less than 10,000. With concentrations of mitomycin higher than those shown here, the incorporation would be even lower. However, the cell loss as a result of mitomycin treatment would exceed 50%.

When lymphocytes or monocytes treated with these reagents are cultured with untreated lymphocytes, the latter become activated. Since this reaction is inhibited by borohydride, it is probably also mediated by the aldehyde groups (Biniamov, Ramot & Novogrodsky, 1974; Biniamov et al., 1975; O'Brien *et al.*, 1974; Greineder & Rosenthal, 1975). The exact way the reaction is induced is uncertain. Perhaps the aldehyde groups on Ls cross-link with certain molecular groups on Lr to trigger the activation. This interaction constitutes an interesting model of cellular interaction.

In the present paper, we investigated this reaction by using purified cell preparations. These were monocyte-depleted lymphocyte samples, T lymphocytes and B lymphocytes. It was found that monocyte-depleted samples were effective stimulator cells. Therefore, lymphocytes as well as monocytes can serve as stimulator cells. Surprisingly, both the T and B fractions were also effective stimulator cells. This probably indicated that aldehyde groups could be induced on the surfaces of both subpopulations.

When T and B fractions were used as responder cells, only the former was active. This selective activa-

tion resembled that obtained with murine lymphocytes. In this species, only the T lymphocytes can be activated by these reagents (Thurman, Giovanella & Goldstein, 1974; Mclan, Wang & Edelman, 1975; Novogrodsky, 1974). There are several possibilities to account for the fact that B lymphocytes can be active as stimulator cells but not as responder cells. The first is that the B lymphocytes do not possess the molecular groups that can cross-link with the aldehyde groups on the stimulator cells. The second possibility is that although the molecular interaction is present, B cells do not have the intracellular mechanism to respond to this surface signal. The present experiment cannot distinguish these possibilities.

We also investigated the effect of the corticosteroid preparation methylprednisolone on this reaction. Very significant inhibition was observed at $10^{-7}$ M of the drug. This concentration can be reached by administering the usual pharmacological doses *in vivo* (Webel *et al.*, 1974). Similarly, this concentration was able to suppress the response of lymphocytes to direct stimulation by solutions of NGAO.

When the NGAO treated lymphocytes were cultured for 48 h, their stimulating ability became much diminished. This is in agreement with the report of O'Brien *et al.* (1974) with $NaIO_4$-treated cells. Presumably either the lymphocytes have lost the stimulating molecular groups or the surface topography has changed so that they no longer react with the responder lymphocytes. Surprisingly the presence of methylprednisolone failed to prevent this loss. This indicated that corticosteroids would not suppress all the activities of the lymphocytes. Certain cell-surface changes could still take place in their presence.

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