vated vaccine no significant protection could be achieved, unless high doses ($10^{10}$ cells) were given on 10 consecutive days. Vaccination with inactivated vaccine on 3 consecutive days yielded no success. After receiving the live vaccine a significant number of animals survived (p 0.001) (table, fig.). No difference was found in the immunogenic effect between the 2 strains of Gal E mutants of *S. typhimurium*. Regarding the inactivated vaccine there was no difference between a vaccine inactivated at 100 °C and the one inactivated at 56 °C. In the table therefore only the term inactivated vaccine is used. 10% of the Balb/C- and C-57-mice, who had died, could by examined for *S. typhimurium:* in 2/3 of these animals Salmonellae could be isolated from liver and spleen.

*Discussion.* Our results confirm previous findings that a live oral vaccine of *S. typhimurium* protects mice against a subsequent challenge with virulent germs[5,9–12]. Live vaccine is superior to inactivated vaccine, unless the latter one is given in high doses on 10 consecutive days[4,6]. Our animal model is a simple method to study the spread of infections among a given population. Vaccinations can be tested under more natural conditions. The individual animal is not submitted to a single heavy dose of the challenging agent but exposed continuously to the infective agent. This model has been applied with equally good results to other infections among rabbit (rabbit-pox) and mice (mouse-pox)[13].


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## Suppression of PHA-induced lymphocyte blastogenesis by concomitant presence of PPD in the culture[1]


N. Aoki and L.J. DeGroot

*Department of Medicine, Kinki University School of Medicine, Osaka (Japan), and Department of Medicine, University of Chicago (Illinois, USA), 22 December 1980*



*Summary.* PHA-induced lymphocyte blastogenic response was remarkably suppressed by the simultaneous presence of PPD in cultures of lymphocytes derived from individuals sensitized to PPD, but not affected by the presence of PPD when the cultures contained lymphocytes from an individual not sensitized to the protein. This double stimulation blastogenesis study with PHA and a specific antigen is feasible as a simple and rapid test to measure cell-mediated immunity to the antigen.


It has recently been demonstrated[2,3] that suppressor cells are generated during in vitro culture of peripheral mononuclear cells (PMC) with purified tuberculoprotein derivative (PPD), leading to a marked regulation of the final expression of PPD-induced blastogenic response in the culture. The generation of such PPD-induced suppressor cells is limited to cultures employing PMC derived from individuals sensitized to the protein. Dose-dependent elevation of suppressor activity following PPD stimulation is in parallel to the blastogenic response to PPD in the same culture. Our previous work revealed[2] that suppressor cell generation in PPD-stimulated PMC cultures is measurable very early – within 40 h, or even after 24 h of incubation; and that suppressor cells once generated act nonspecifically to reduce the blastogenic response of PMC to phytohemagglutinin-P (PHA) as well as PPD. It was therefore naturally expected that DNA synthesis in a PHA-driven PMC culture would be considerably affected by the concomitant presence of PPD in the cultures. Such antigen-activated suppressor activity in the PPD and PHA double stimulation cultures would be most evident when DNA synthesis was measured at day 3 of culture, considering maximal blastogenic response to PHA, and in contrast, the negligible contribution of PPD-induced DNA synthesis to the total response in the cultures at this time. The present study was undertaken to confirm the early inhibitory effects exerted by the concomitant presence of PPD (specific antigen) on PHA-induced lymphocyte blastogenic response. If such a double stimulation assay can detect the early generation of suppressor cells associated with antigen specific lymphocyte responses, it might offer another simple and rapid test to measure cell-mediated immunity.

*Materials and methods.* 3 subjects who were differently sensitized to PPD, as assessed by tuberculin skin reaction, were examined for the present study. Subject A and subject B showed strong and weak skin test responses, respectively, whereas subject C gave a negative response. Conventional in vitro blastogenesis studies using an optimal concentration of PPD (2.5 µg/ml) as described previously[2] gave stimulation indices of 24.0, 3.5 and 0.9 for subjects A, B and C, respectively, at day 7 of culture. PMC were separated on Ficoll-Paque (Pharmacia) from heparinized blood and cultured in F-10 culture medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO) at 37 °C in 5% $CO_2$ in moist air. The PMC were cultured in 6–8 multiplicates in microculture plates (Falcon), each well containing $8 \times 10^4$ cells in 0.2 ml of culture medium with or without mitogens. As mitogens, PPD (Parke-Davis) and PHA (Difco) were used. Optimal dose levels for maximal blastogenic response in the present culture system were 2.5 µg/ml for PPD and a 1:100 dilution for PHA[2]. For the double-stimulation studies, various doses of PPD ranging 0.0025 µg/ml to 2.5 µg/ml and a constant suboptimal dose of PHA at a final dilution 1:1000 were used as shown in table 1. The advantage of using a suboptimal dose of PHA to appreciate minimal generation of suppressor activity in PMC cultures was described previously[4]. PHA was added to the cultures simultaneously with PPD, or 20 h after addition of PPD. In every case, DNA synthesis was measured exactly 72 h after the addition of PHA. The cells were pulsed with $^{125}$I-

Table 1. Suppression of PHA-induced lymphocyte blastogenesis in the presence of PPD, with a 20-h delay in PHA addition to PPD-including culture

| | Protein concentration (μg/ml) in double-stimulated culture | | | | | |
|---|---|---|---|---|---|---|
| | No PPD (control) | PPD (0.0025) | PPD (0.025) | PPD (0.25) | PPD (2.5) | OVA (2.5) |
| Subject A | | | | | | |
| 1. PHA, 1:1000 | 11192±557* | 11463±76 | 10524±239 | 9597±483 | 7304±619 | 11788±176 |
| | | NS** | $p<0.05$ | $p<0.01$ | $p<0.001$ | NS |
| 2. No PHA | 48±25 | 57±6 | 153±22 | 344±38 | 496±8 | 53±28 |
| Subject B | | | | | | |
| 1. PHA, 1:1000 | 9671±541 | 9877±451 | 9626±757 | 8840±301 | 7959±533 | 9593±678 |
| | | NS | NS | $p<0.05$ | $p<0.01$ | NS |
| 2. No PHA | 100±90 | 87±25 | 91±33 | 158±57 | 227±79 | 80±51 |
| Subject C | | | | | | |
| 1. PHA, 1:1000 | 9807±627 | | | 9811±174 | 9602±545 | 10015±799 |
| | | | | NS | NS | NS |
| 2. No PHA | 88±35 | | | 101±44 | 94±44 | 97±27 |

1. 92-h culture mixed with PPD alone for the initial 20 h, subsequently with PHA added for further 72 h, pulsed with 0.5 μCi of $^{125}$I-IUdR for the last 4 h. 2. 92-h culture without PHA addition, pulsed with 0.5 μCi of $^{125}$I-IUdR for the last 4 h. * cpm (mean±SD for 6 cultures). ** Comparison to control (Student's t-test). NS: not significant. OVA: ovalbumin (Sigma).

Table 2. Suppression of PHA-induced lymphocyte blastogenesis in the presence of PPD, with simultaneous addition of PHA and PPD at the initiation of culture

| | Protein concentration (μg/ml) in double-stimulated culture | | | | | |
|---|---|---|---|---|---|---|
| | No PPD (control) | PPD (0.0025) | PPD (0.025) | PPD (0.25) | PPD (2.5) | OVA (2.5) |
| Subject B | | | | | | |
| 1. PHA, 1:1000 | 7230±726* | 7100±555 | 6934±565 | 6872±540 | 6085±650 | 6940±637 |
| | | NS** | NS | NS | $p<0.05$ | NS |
| 2. No PHA | 108±65 | 105±72 | 115±59 | 147±76 | 210±18 | 112±28 |

1. 3-day culture simultaneously stimulated with PPD and PHA, pulsed with 0.5 μCi of $^{125}$I-IUdR during the last for 4 h. 2. 3-day culture without PHA addition, pulsed with 0.5 μCi of $^{125}$I-IUdR during the last 4 h. * cpm (mean±SD for 8 cultures). ** Comparison to control (Student's t-test).

iododexyuridine ($^{125}$I-IUdR NEN, 0.5 μCi per well) for the last 4 h of culture and harvested on glass wool filters using an automated cell harvester. Incorporation of $^{125}$I-IUdR into DNA was measured using a gamma counter.

*Results and discussion.* When various doses of PPD were introduced into PMC cultures 20 h prior to the addition of PHA, the PHA-induced blastogenic response in the cultures evaluated 3 days after PHA addition was found to be significantly suppressed in subjects A and B who were sensitized to PPD but not in subject C who was not sensitized to the protein (table 1). Ovalbumin (control protein), showing no blastogenic response in any of the subjects, could not substitute for PPD in the induction of suppressive effects on PHA-induced blastogenic response in subjects A and B. It is quite natural to suppose that these PPD-induced suppressive effects are based on suppressor cells generated in vitro as shown previously[2]. It is clearly shown in the data from subject A that there is a dose-related induction of suppressor activity dependent upon PPD concentrations in the double-stimulated cultures. Although 3–4 days of culture in the present culture system were too short for evaluation of PPD-induced lymphocyte blastogenesis which usually takes at least 7 days of culture for full expression to take place, slight but significant rises in blastogenic response attributable to PPD-induced blastogenesis were observed in the cultures not including PHA. It should be noted that the PPD-induced rise in DNA synthesis in PMC never contributed an additive increase in the total magnitude of DNA synthesis in the cultures driven by both PPD and PHA; conversely, the suppressor cells generated in the same culture reduced the magnitude of blastogenesis in the double-stimulation cultures by overcoming PPD-induced blastogenic effects. Similar PPD-induced suppression was also noted in PMC cultures to which PPD and PHA were simultaneously added at the initiation of incubation (table 2), although less suppressor activity was generated in the simultaneous double-stimulation study as compared to the activity exhibited in the PMC cultures to which PHA was added with a delay of 20 h after PPD addition. In repeated experiments dealing with 4 other individuals who showed weak sensitization to PPD as in subject B, the simultaneous double-stimulation study with PPD and PHA always gave a statistically significant reduction in lymphocyte blastogenesis at a dose level of 2.5 μg/ml for PPD as compared with the response in cultures stimulated solely with PHA, but no reduction in the response when the same dose of OVA was substituted for PPD (data not shown).

Thus, based on the previous work which showed the early generation of suppressor cells in PPD-stimulated PMC cultures, the present study has shown a simple culture technique which identifies antigen-activated suppressor activity by utilizing a combination of PHA, a potent and nonspecific mitogen, and PPD, a specific antigen with weaker mitogenicity, as stimulants. Although the findings observed appear to be involved in antigenic competition or antigen-activated nonspecific immune suppression[5–7], which usually requires in vivo or in vitro preexposure of lymphoid cells to an antigen for more than 3 days to manifest the effect, the present results indicate that PPD-induced suppressor activity is effective even during the 1st day of culture, thereby interplaying with the stimulatory drive exerted by PHA and PPD throughout the culture. In addition, the double stimulation study, described here as an example of suppressor cell generation, allows a more rapid detection of the immune response elicited in PMC cultures by a specific antigen than does the conventional lymphocyte blastogenesis assay. Namely, the present technique

might be possible as another method useful for the detection of cellular immunity to defined antigens, although so far its usefulness is limited to PPD.

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## Cortisol resistant RPMI-1788 lymphocytes become sensitive to cortisol subsequent to a 24-h incubation period in medium containing purified human transcortin[1]

L. Amaral and S. Werthamer

*Department of Pathology, The Bronx-Lebanon Hospital Center, 1276 Fulton Avenue, Bronx (New York 10456, USA), and Department of Pathology, Methodist Hospital of Brooklyn, Brooklyn (New York 11215, USA), 20 January 1981*

*Summary.* RPMI-1788 lymphocytes (a human cell line) are resistant to cortisol in vitro. Prior incubation for a minimum of 24 h in a medium which contains purified human transcortin at a concentration of 50 µg/ml renders these cells sensitive to the inhibitory action of cortisol as regards the synthesis of DNA. Only the transcortin-exposed cells contain a cortisol binding species whose sedimentation behavior in a sucrose gradient is identical to that of transcortin.

The response of a target cell to a steroid is predicated upon the existence of a steroid-specific, cytoplasmic receptor protein that is physiologically active[2]. Some cortisol responsive cells of man contain cortisol-binding proteins that exhibit physio-chemical and immunological characteristics that are similar to human transcortin[3-5]. The role of this transcortin-like protein has been postulated to be that of a receptor[3].

RPMI-1788 lymphocytes continuously cultured in our laboratory were washed 3 times in serum-free, TC199 medium each wash being followed by centrifugation at $850 \times g$ for 20 min at 4 °C. The pellet of cells was suspended in cold medium and the concentration of cells adjusted to $5 \times 10^5$/ml. To duplicate flasks containing 25 ml of the cell suspension, heat-denatured fetal calf serum was added to make a final concentration of 15%. To one of the flasks, lyophilized purified transcortin[3] was added to yield a final concentration of 50 µg/ml. The flasks were incubated for 24 h, the cultures were centrifuged and the cell pellets washed 3 times with ice cold TC 199 medium. The last pellets were suspended in ice cold TC 199 medium to yield a cell count of $5 \times 10^5$/ml. Aliquots of 1 ml from each flask were transferred to separate triplicate tubes containing 50 µl of the vehicle used for the preparation of cortisol[7]. To each culture 1 µCi of $^3$H-thymidine (New England Nuclear Corp.) sp. act. of 22.4 Ci/mm was added and the cultures incubated for 2 h at 37 °C. The cpm of radioactive thymidine incorporated by each culture were obtained by methods previously described[5]. The percent deviation from the mean cpm of the triplicate set did not exceed 5%.

The effect of cortisol on the incorporation of $^3$H-thymidine by RPMI-1788 lymphocytes cultured in the absence and presence of purified human transcortin

| Prior 24 h incubation with 15% fetal calf serum | cpm of $^3$H-thymidine/$10^5$ cells ± SD | Difference |
|---|---|---|
| Control | 129,590 ± 835 | - |
| Cortisol $10^{-8}$ M | 131,250 ± 1040 | +2% |
| Cortisol $10^{-7}$ M | 130,400 ± 880 | 0% |
| Cortisol $10^{-6}$ M | 130,280 ± 650 | 0% |
| Cortisol $10^{-5}$ M | 129,890 ± 810 | 0% |
| **Prior 24 h incubation with 50 µg of transcortin/ml of medium and 15% calf serum** | **Average cpm of $^3$H-thymidine/$10^5$ cells ± SD** | **Difference** |
| Control | 97,440 ± 935 | - |
| Cortisol $10^{-8}$ M | 96,050 ± 1090 | −2% |
| Cortisol $10^{-7}$ M | 72,910 ± 625 | −25% |
| Cortisol $10^{-6}$ M | 59,630 ± 710 | −39% |
| Cortisol $10^{-5}$ M | 41,380 ± 470 | −58% |

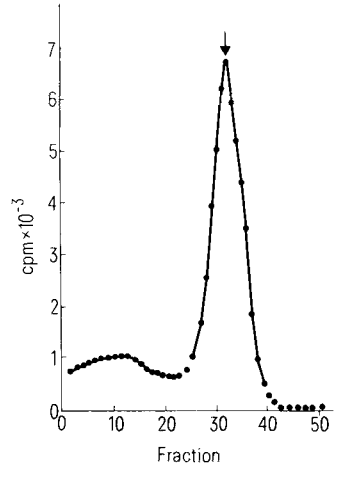


Fig. 1. Sucrose gradient sedimentation of cortisol binding species of cytosols obtained from cortisol-responsive (transcortin exposed) an cortisol-unresponsive RPMI-1788 lymphocytes. Cytosols were prepared from $10^8$ transcortin-exposed and unexposed RPMI-1788 cells by procedures previously described[2]. Aliquots of 1 ml containing approximately 1 mg of protein were incubated with $10^6$ cpm of $^3$H-cortisol for 1 h at 4 °C. At the end of this period, 1 mg of dextran-coated charcoal was added to each tube, the tubes incubated for 1 h in a shaking water bath maintained at 4 °C and then centrifuged at $6000 \times g$ for 20 min. Duplicate aliquots of 0.3 ml were layered on top of 5-20% sucrose gradients and centrifuged for 21 h at 4 °C at 60,000 rpm in a Beckman SW-65 rotor. The gradients were fractionated as previously described[3]. (The arrow denotes the fraction coresponding to the peak cpm of cortisol bound by purified transcortin.)