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Comparison of murine B-cell proliferative response to bacterial lipopolysaccharide and DNP derivative of *Mycobacterium tuberculosis* antigens

ANILA PRABHU and R K SAXENA
School of Life Sciences, Jawaharlal Nehru University, New Delhi 110 067, India

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Abstract. The DNP derivative of sonicate antigens of the H37Ra strain of *Mycobacterium tuberculosis* (Ra-DNP) is known to induce marked B-cell proliferation. In order to understand whether B-cell proliferation in response to Ra-DNP was antigen driven or represented a non-specific mitogenic effect of Ra-DNP, the effect of Ra-DNP was compared with that of lipopolysaccharide a potent B-cell mitogen. Parameters used for comparison were (i) thymidine incorporation, (ii) viable cell counts, (iii) amount of Ig secreted, (iv) isotype profile of Ig released and (v) cell cycling pattern of B-cells in culture. Overall the effect of Ra-DNP was found to be essentially similar to that of lipopolysaccharide for all parameters examined. Yet quantitatively, the effect of the former was always relatively poorer. At optimal doses, the effect of Ra-DNP ranged from 50 to 70% of the lipopolysaccharide effect in different assays. These results suggest that Ra-DNP may have a B-cell mitogenic effect similar to the effect of lipopolysaccharide, but all B-cells may not respond to Ra-DNP.

Keywords. *Mycobacterium tuberculosis;* lipopolysaccharide; DNP; B-cells; mitogens.

1. Introduction

Sonicate antigens of *Mycobacterium tuberculosis* are a complex mixture of proteins, carbohydrates and lipids present in free or complex form (Grange 1984; Petit and Lederer 1984) and are capable of inducing specific humoral as well as cell mediated immune responses (Daniel and Janicki 1978). Non-specific effects of a variety of bacterial products on immune response have also been known for a long time. Bacterial lipopolysaccharide (LPS) are potent B-cell mitogens (Anderson *et al* 1973). In addition several preparations of bacterial origin have non-specific adjuvant effect on specific immune response (Messina *et al* 1991).

Recently we have shown that DNP derivatives of *M. tuberculosis* (H37Ra strain) sonicate antigen (Ra-DNP) induce marked proliferative response in spleen and lymph node cells from unsensitized mice (Prabhu *et al* 1993a). Underivatized Ra antigen however induced little proliferation activity. Using fractionated cell preparations it was found that the proliferative response was essentially restricted to B-cells. Even though unsensitized mice were used in the study, it was not clear whether the proliferative response was antigen driven or mitogen driven. In the present study we have further examined the Ra-DNP induced B-cell proliferative response and compared it to the effect of LPS. Our results indicate that the characteristics of

Ra-DNP induced B-cell proliferative response are similar to a mitogenic response, although the magnitude of the response is low when compared to the effect of LPS.

2. Materials and methods

2.1 *Animals*

In all experiments 8-12 weeks old C57B1/6 mice were used. Animals were bred in the animal house facility of Jawaharlal Nehru University and were fed standard mouse pellet diet from Hindustan Lever.

2.2 *H37Ra antigen preparation*

A soluble preparation of the H37Ra strain of *M. tuberculosis* antigen was prepared as described before (Udaykumar *et al* 1991). Briefly, the strain was cultured for 3 weeks on Sauton's medium. Cells were harvested by centrifugation and washed thrice in PBS (pH 7.2). This was followed by 9 freeze-thaw cycles using liquid nitrogen and warm water and the suspension was subjected to sonication in an MSE ultrasonicator. The sonicate was centrifuged at 1,40,000 *g* for 1 h at 10°C and aliquots of the supernatant were stored at –70°C (Ra antigen). DNP derivatives of the antigen was prepared by the method of Sanger (1945). Briefly, Ra antigen (5 mg/ml) was dialyzed against 0.3 M sodium-bicarbonate (pH 8·1) and incubated with 1 mg of fluoro-2,4-dinitrobenzene (FDNB, stock 100 mg/ml in ethanol) for 2 h at room temperature. After incubation, the preparation was dialyzed extensively against PBS (pH 7·2), filter sterilized and stored at – 70°C till used (Ra-DNP).

2.3 *Cell preparation and culture conditions*

Single cell suspensions from spleen of unsensitized mice were prepared as described previously (Sarin and Saxena 1989). Cell preparation was washed thrice in PBS (pH7.2) and suspended in RPMI 1640 supplemented with 10% FCS, 2×10^{-5} M 2 ME, 300 μg/ml glutamine and 60μg/ml gentamycin (complete medium). Trypan blue excluding cells were counted using a haemocytometer. Cells were cultured at $2{\cdot}5 \times 10^6$ cells/ml in 0.2 ml of complete medium with or without various test antigens in 96 well flat bottom microtest plate in triplicate. After required time duration, proliferative activity was determined by giving an 18 h pulse with 0.5 μCi of tritiated thymidine as described before (Prabhu *et al* 1993b).

2.4 *ELISA for secretory immunoglobulin and isotype of secretory immunoglobulin*

Estimation of secretory immunoglobulin (Ig) in supernatants of mouse spleen cells cultured with Ra and Ra-DNP was done by ELISA. Affinity purified rabbit anti-mouse Ig was coated on an ELISA plate (1 μg/ml, overnight). After blocking the wells with 3% BSA, appropriate dilution of supernatant was added and incubated for 2 h at 37°C. Plates were washed and bound mouse Ig was reacted with rabbit

anti-mouse Ig-HRPO for 2 h at 37°C. After washing the plates, 0.1 ml substrate (6 mg OPD in 12 ml citrate buffer with 10µl of 30% H_2O_2) was added to the assay wells. The reaction was stopped using 5 N H_2SO_4 and absorbance was read at 495 nm (ELISA reader, Biotek). Isotype profile of Ig secreted by murine spleen cells cultured with Ra, Ra-DNP and LPS was determined by ELISA using a monoclonal antibody identification kit (Zymed).

2.5 *Cell cycling analysis*

Cell cycle analysis was done on sorted B-cell populations by staining the cells with propidium iodide and analysing the stained cell preparations on a Becton Dickinson FACSTAR flowcytometer as described before (Gulberg and Smith 1986; Saxena *et al* 1989).

3. Results

3.1 *Comparison of effect of Rα, Ra-DNP and LPS on proliferative activity of mouse spleen cell preparations*

Ability of Ra, Ra-DNP and LPS preparations to induce proliferative response in murine spleen cells was compared at various doses. Results of a representative experiment in figure 1 indicate that the underivatized Ra antigen induced only a marginal proliferative response. Ra-DNP induced a proliferative response which was dose dependent up to 8 µg/ml and plateaued thereafter at a level of about 24,000 cpm/well. Lipopolysaccharide (LPS) induced a maximal response even at the lowest test dose of l00 ng/ml at a level of about 33,000 cpm/well. Optimum response to Ra-DNP therefore remained significantly lower than that of LPS. Time kinetics of effect of Ra, Ra-DNP and LPS were also compared using as parameters thymidine incorporation, viable cell counts and release of antibody by cultured spleen cells. Results in figure 2 indicate that both Ra-DNP and LPS induce maximum thymidine incorporation on day 1. An increase in viable cell counts in case of Ra-DNP was observed between day 3 and 6. Ra antigen did not affect cell numbers significantly. LPS induced the largest increase in cell number and this was observed between day 2 and 5. Antibody secretion peaked on day 6 or 7 for Ra, Ra-DNP and LPS. In all the parameters utilized, the magnitude of the response to LPS was significantly higher than that to Ra-DNP.

Besides Ra-DNP, DNP derivatives of some other antigen preparations were also examined for their proliferation inducing effect on unsensitized spleen cells. While BSA-DNP had no effect on mouse spleen cells, DNP derivative of *E. coli* sonicate antigen had significantly better effect than underivatized *E. coli* antigen preparations. In a typical experiment, DPM of thymidine incorporations in Ra and Ra-DNP treated cultures were 15,523 and 32,251 respectively (107% increase), whereas corresponding values for control and DNP derivatized *E. coli* preparations were 32,567 and 59,103 (81% increase). Enhanced mitogenic activity as a result of DNP derivatization is thus not specific to Ra antigens.

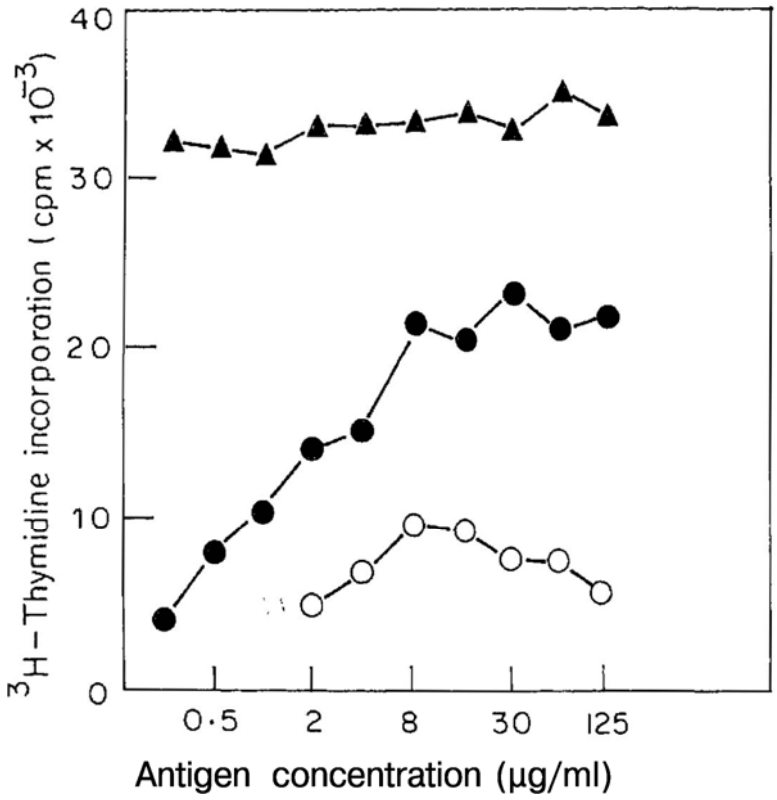

Figure 1. Comparison of mitogenic effect of LPS, Ra-DNP and Ra. Spleen cells were cultured with different concentrations of Ra (O), Ra-DNP (●) and LPS(▲) Proliferative activity was measured 24 h after initiation of culture by thymidine incorporation, as described in §2.

3.2 *Isotype of secreted Ig in supernatant of spleen cells cultured with Ra, Ra-DNP and LPS*

Isotype profile of Ig secreted by spleen cells cultured with Ra, Ra-DNP and LPS was examined. Results in figure 3 indicate that in all cases µ, α and γ-2 heavy chains and k light chains were predominantly represented in the secreted Igs. Ra-DNP as well as LPS appear to induce a relatively greater release of IgG_3 isotype as compared to control and Ra treated cultures.

3.3 *Cell cycle analysis of B-cells cultured with Rα, Ra-DNP and LPS*

Cell cycle analysis was done on positively sorted B-cells. Mouse spleen cells were stained with a rabbit anti-mouse Ig antibody-FITC preparation and positively selected for B-cells. A comparison of flowcytometric profiles of pre- and post-sorted cells is shown in figure 4. Results in figure 4B indicate that the purity of sorted B-cells was above 99%. Sorted B-cells were cultured for 24 h with or without different antigen preparations and were stained with propidium iodide for an analysis of cell

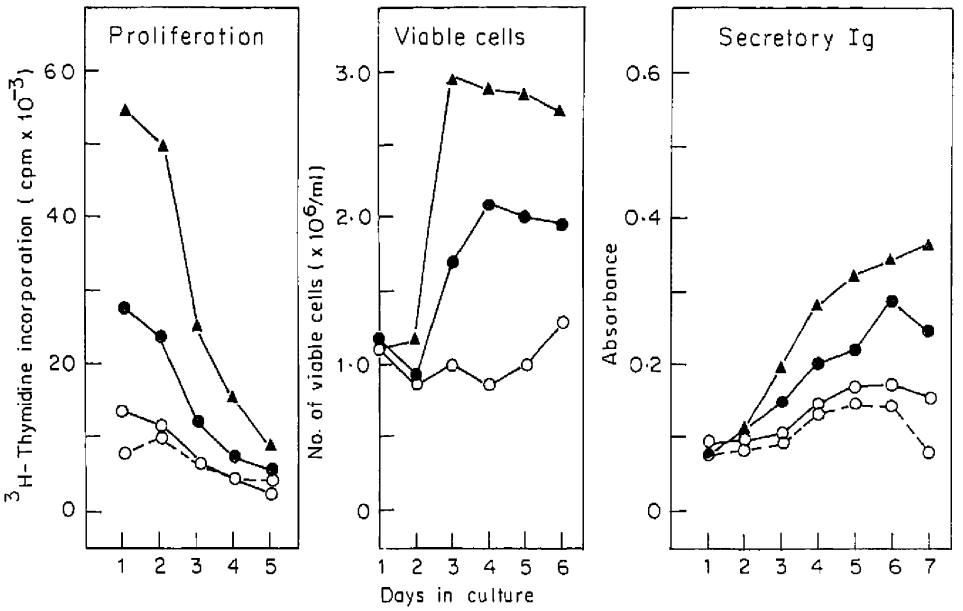

Figure 2. Time kinetics of effect of Ra, Ra-DNP and LPS. Mouse spleen cells were cultured without antigen (…O…)or with 20 μg/ml of Ra (O), Ra-DNP(●)and LPS (▲) in triplicate sets. At each time point, thymidine incorporation, viable cell number and levels of secretory Ig in culture supernatant was determined as described in §2.

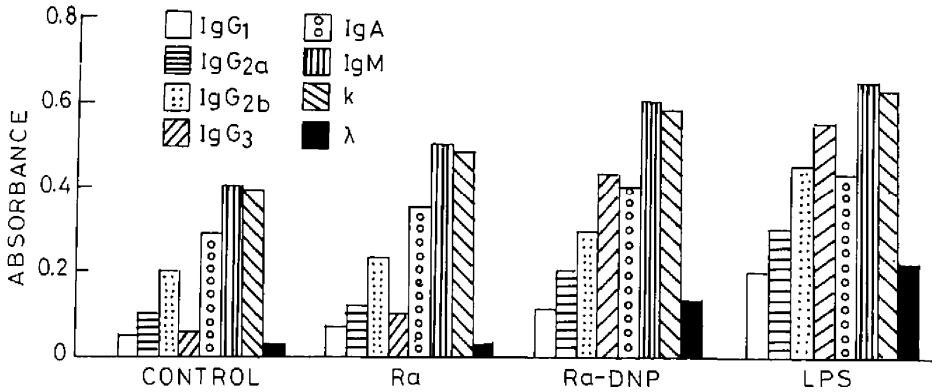

Figure 3. Isotype profile of secreted lg in supernatant of spleen cells cultured with Ra, Ra-DNP and LPS. Culture supernatant from unstimulated spleen cells and cells cultured with Ra, Ra-DNP and LPS, were harvested and relative levels of antibodies with different isotypes were determined by ELISA.

cycle pattern on a flowcytometer. Results in figure 5 show that freshly sorted and uncultured cells were essentially in G_0/G_1 (resting) phase with less than 1% of the cells in the S-phase. B-cells cultured alone or with Ra antigen had 12 and 13% of the cells in S-phase respectively. Both Ra-DNP and LPS induced a significant increase in the proportion of cells in the S-phase (23% and 41% respectively), although effect of LPS was more pronounced in this respect.

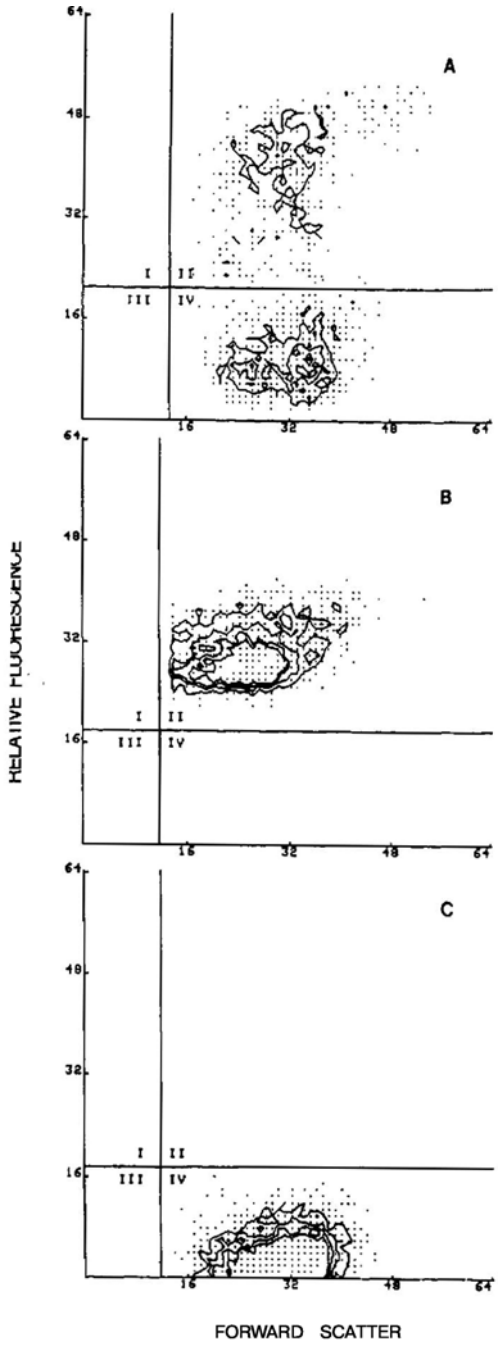

Figure 4. B-cell purification by cell sorting. Mouse spleen cells were stained with a rabbit anti-mouse Ig antibody-FITC preparation. **(A)** Staining profile of spleen cells in which Ig-positive B-cells are in II quadrangle and unstained non-B-cells in quadrangle IV. After sorting the cells, the positively and negatively selected cell preparations were reanalysed. **(B)** Pure B-cells (purity above 99%) which were used in cell cycling experiments (figure 5). **(C)** Sorted non-B-cells.

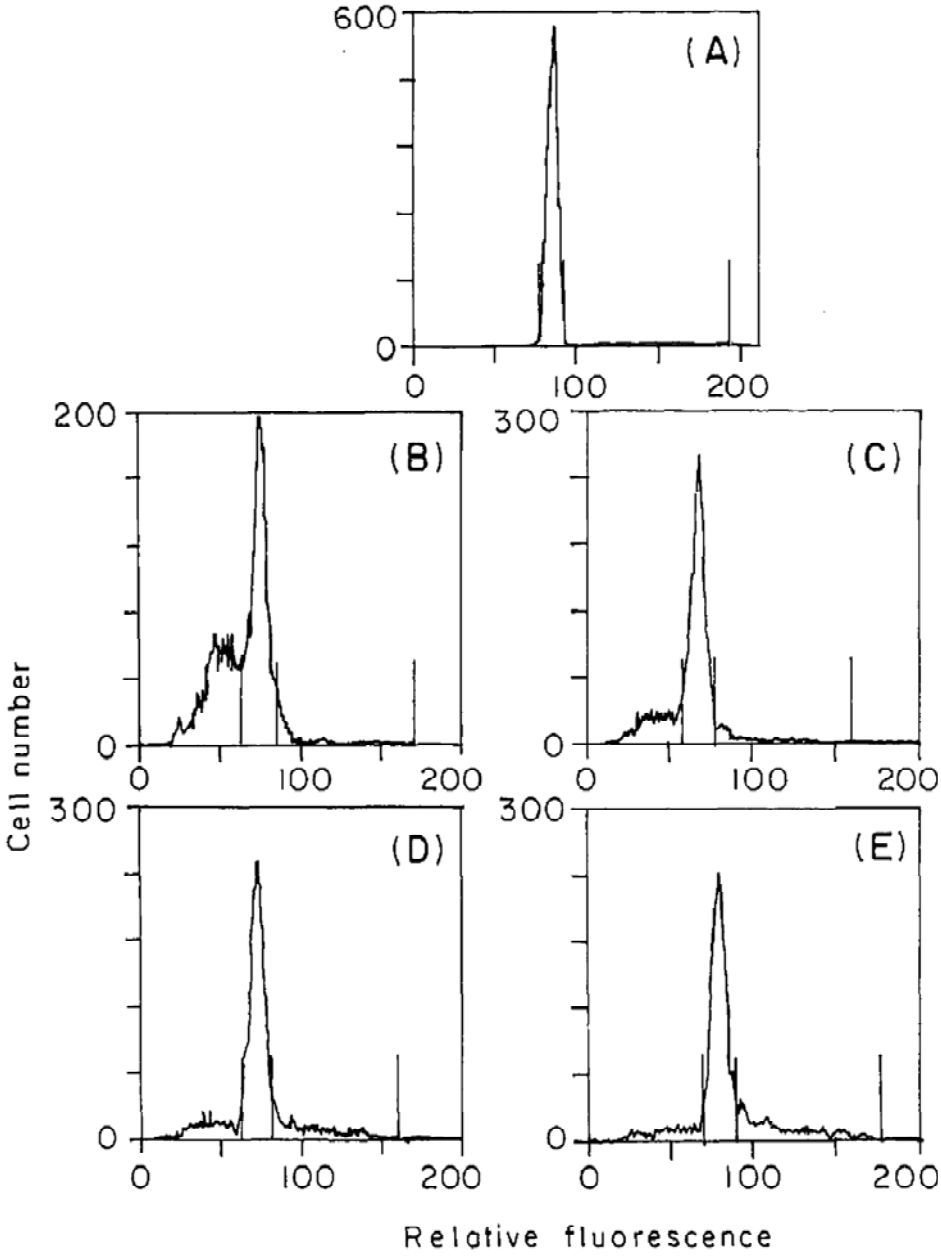

Figure 5. Cell cycle analysis of B-cells cultured with Ra, Ra-DNF and LPS. Sorted B-cells were cultured in 24 well plate without antigen or with 20 μg/ml of Ra, Ra-DNP or LPS for 3 days. Cultured cells were washed, stained with propidium iodide for DNA and analysed for cell cycle pattern using a flowcytometer as described in methods. Cell cycle pattern of freshly sorted B-cells **(A),** B-cells cultured without antigen **(B)** and B-cells cultured with Ra **(C),** Ra-DNP **(D)** and LPS **(E)** are shown in the figure. Percentage of cells in G_0/G_1, S and G_2/M phases respectively were as follows: 99, 1, 0 **(A);** 87, 12, 1 **(B);** 87, 13, 1 **(C);** 76, 23, 1 **(D);** 56, 41, 3 **(E).**

4. Discussion

We have previously shown that the DNP derivative of sonicate antigens of *M. tuberculosis* (Ra-DNP) induces a marked proliferative response in unsensitized murine B-cells (Prabhu *et al* 1993a). Underivatized Ra antigen as Dell as DNP derivative of proteins like BSA and rabbit-Ig had no such effect on B-cells (Prabhu *et al* 1993). A LPS contamination of Ra-DNP preparation as an explanation for the proliferative effect of the latter appears unlikely since that would not explain the lack of similar effect in the non-derivatized Ra preparation. Moreover, affinity columns capable of absorbing LPS did not absorb the B-cell proliferative activity in Ra-DNP preparations (results not shown). DNP derivatization of Ra antigen may generate unique B-cell stimulating determinants in Ra-DNP preparations. The B-cell proliferative response could in that case represent a clonal expansion of B-cells specifically recognizing the stimulatory determinants on Ra-DNP. Alternatively, new determinants generated by DNP derivatization may induce a polyclonal B-cell response similar to a LPS response in B-cells.

The present investigation reveals that even though the kinetics of effect of LPS and Ra-DNP (on thymidine incorporation, viable cell count as well as on the amount and isotype profile of Ig secreted by spleen cells) were very similar, the overall effect of Ra-DNP was significantly weaker than that of LPS. LPS is a potent B-cell mitogen (Anderson *et al* 1973) and at optimum dose it is expected to induce proliferation in all B-cells irrespective of their antigenic specificity. In [^{3}H]thymidine incorporation experiments, the Ra-DNP effect on proliferation plateaued at a level about 70% of the optimal effect of LPS. This indicates that Ra-DNP, even at optimal dose, may not induce proliferation in all B-cells. This point is further supported by our cell cycling studies in which 41 % of B-cells cultured with optimum dose of LPS, were in DNA synthetic phase. Only 24% of the Ra-DNP treated cells were in S-phase. A significantly lower but comparable proportion (about 12%) of control and Ra treated cultured B-cells were also found to be in S-phase, which could have happened due to activation with fetal calf serum factors or antigens.

It might be argued that Ra-DNP is intrinsically less efficient than LPS as an activator of B-cells. However, both the [^{3}H]thymidine incorporation results and the cell cycle data argue against this. Rather, we suggest that Ra-DNP may induce proliferation in a subpopulation of B-cells. From the differences observed in the magnitude of the optimal response to Ra-DNP and LPS, it is likely that about half of all B-cells respond to Ra-DNP. If Ra-DNP's effect was antigen specific, one would have expected to see a much lower proportion of B-cells pushed into the S-phase (because B-cells specifically recognizing Ra-DNP as antigen are unlikely to constitute 50% of all B-cells). We therefore suggest that Ra-DNP acts as a non-specific B-cell mitogen. Due to reasons not clear at present, it can induce a proliferative response in a large proportion of B-cells but not in all. It would be interesting to study the differences in B-cell populations which are responsive or non responsive to Ra-DNP. Work in our laboratory is continuing in this direction.

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