

## IN VITRO CELL ACTIVATING PROPERTIES OF THE COMPOSITE RIBOSOMAL VACCINE D53

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*D53 (Ribomunyl<sup>R</sup>) is a composite vaccine made of immunogenic ribosomes from 4 bacterial species (Klebsiella pneumoniae, Haemophilus influenzae, Streptococcus pyogenes and Streptococcus pneumoniae) associated with a membrane proteoglycan from a non encapsulated strain of Klebsiella pneumoniae. D53 is a potent inducer of interleukin-1 production by mouse BALB/c spleen cells as shown by the C3H/HeJ thymocyte co-stimulation assay. Furthermore D53 triggers DNA synthesis by mouse spleen cells and induces the maturation of B lymphocytes into immunoglobulin secreting cells. Polyclonal B cell activation by D53 was readily achieved in the C3H/HeJ strain which is deficient in its response to E. coli lipopolysaccharide. The proliferative response to D53 was abrogated by removal of B cells from the spleen cell suspension, but it was not altered after depletion of T cells or adherent cells. D53 induced polyclonal B cell activation of spleen cells from athymic nude mice and from CBA/N mice. Each component of D53 induced polyclonal B cell activation except ribosomes from Streptococcus pneumoniae. Each triggered Interleukin-1 synthesis except ribosomes from Klebsiella pneumoniae. These in vitro properties may account for some of the in vivo immunostimulating properties of this composite vaccine.*

D53 (Ribomunyl<sup>R</sup>, Pierre Fabre SA, Castres, France) has been extensively used in the prevention of recurrences of respiratory tract infections (Michel et al., 1978; Bousquet & Dussourd d'Hinterland, 1982). It is a composite vaccine (Dussourd d'Hinterland et al., 1980) which contains a membrane proteoglycan (MPG) of a peculiar non-encapsulated non-virulent strain of *Klebsiella pneumoniae* (*K.p.*) (biotype a, I-145) associated with ribosomes from 4 bacterial species: *K.p.*, *Haemophilus influenzae* (*H.i.*), *Streptococcus pyogenes* (*St. pyo.*) and *Streptococcus pneumoniae* (*St. pn.*). D53 was reported to be immunogenic in various species including man and mouse and to induce the production of protective antibodies (Pinel et al., 1985a,b). Furthermore *K.p.* MPG, the major component of D53, was shown to enhance NK cell activity (Dussourd d'Hinterland et al., 1985). Therefore D53 may be considered not only as a composite vaccine but also as an immunomodulating agent. In this respect, we have investigated the capacity of D53 and that of each of its components to induce: (i) the production of interleukin-1 (Il-1) by mouse macrophages, (ii) the proliferation of resting B lymphocytes and (iii) their maturation into immunoglobulin secreting cells.

### MATERIAL AND METHODS

**Animals** — Adult (5-10 week-old) male and female C3H/HeN, C3H/HeJ and nu<sup>+</sup>/nu<sup>+</sup> mice were obtained from IFFA-Credo, L'Arbresle, France. BALB/c mice were bred in our laboratory. CBA/N mice were obtained from Dr. Guenet (Institut Pasteur, Paris).

**Reagents** — D53 (Ribomunyl<sup>R</sup>) was kept lyophilised at ambient temperature and reconstituted in pyrogen-free distilled water before use. *K.p.* MPG and ribosomes from *K.p.*, *H.i.*, *St. pyo.* and *St. pn.* were prepared as previously described (Dussourd d'Hinterland et al., 1980). Dilutions were made in culture medium. Phytohemagglutinin (PHA, HA15, Wellcome, Dartford, UK), Concanavalin A (Con A, IBF, France) and *E. coli* Lipopolysaccharide (LPS, 0111 B4, Difco, Detroit, MI) were used at optimal stimulatory concentrations of 90, 5 and 100 µg/ml respectively, according to previous assays. Control unstimulated cultures received phosphate buffered saline (PBS) instead of the mitogen.

**Cell culture** — Spleen cells or thymocytes were collected by gentle teasing and cultured in flat bottom microtiter plates at 4 x 10<sup>5</sup> cells in 0.2 ml. The culture medium (CM) was made of RPMI 1640 supplemented with 1% glutamine, antibiotics and serum-free medium (ADCM 8503, Centre de Transfusion Sanguine,

Lyon, France). Cultures were incubated at 37°C, in a humidified atmosphere with 7% CO<sub>2</sub>. For some cultures Fetal Calf Serum (FCS, Seromed GmbH Munich, FRG) was used at a final concentration of 10% (v/v) instead of ADCM. Thymidine incorporation into nuclei was measured by addition of 2 µCi of <sup>3</sup>H-Thymidine (THM 79A, CEA Saclay, France), 24h before harvesting. Cells were collected on glass fiber filters and radioactivity measured in a scintillation counter. Results in cpm are means of triplicate cultures.

For measurement of Ig synthesis cells were cultured at 2x10<sup>6</sup> in one ml in plastic tubes, in the presence of FCS. After 5 days cell free supernatants were collected and kept frozen.

*Depletion of adherent cells* – Spleen cells were incubated in RPMI 1640 supplemented with 20% heat-inactivated FCS, 90 min, at 37°C, 7% CO<sub>2</sub> in a Falcon<sup>R</sup> plastic dish. After washings, the cells were incubated once more for 60 min at 37°C. Non adherent cells were then recovered. Such suspensions contained less than 1% macrophages as assessed by May-Grunwald-Giemsa staining.

*B and T cell depletion* – Spleen cells were depleted in B or T cells, using the method of Julius et al. (1973). Briefly, spleen cells (1x10<sup>8</sup> cells/ml in RPMI 1640 containing 10% FCS) were passed over a 5ml nylon wool column and incubated for 45 min at 37°C, 7% CO<sub>2</sub>. The first filtrate of the column at 20°C contained approximately 65% Thy1.2 positive cells and 15% surface immunoglobulin (S-Ig) positive cells. Then nylon wool was put into a Petri dish containing RPMI 1640 at 37°C. After 10 min under agitation, a cell suspension containing approximately 10% Thy1.2 positive cells and 70% S-Ig positive cells was recovered.

*B cell enrichment* – Spleen cell suspensions were enriched in B cells, using two different methods: panning (Wysocki & Sato, 1978) and cytotoxicity using anti-Thy 1.2 antibody.

(1) Spleen cells were incubated in Plastic Petri dishes (1029 Falcon Plastics, Oxnard, CA), coated with a mixture of goat anti-mouse IgG, IgA, IgM (Zymed Laboratories, San Francisco, CA), diluted in 2.5 mM veronal buffer pH 8.6. After removal of non adherent cells, adherent cells were recovered with a rubber policeman in RPMI medium containing 5% heat-inactivated fetal calf serum (FCS, Seromed, GmbH, Munich, FRG). These adherent cells

comprised at least 80% S-Ig positive and less than 1% Thy 1.2 positive cells.

(2) In the second method, 10<sup>7</sup> spleen cells were incubated with a 1/300 dilution of anti-Thy 1.2 monoclonal antibody (Cedarlane Laboratories, Hornby, Ontario) for 60 min at 4°C. The cells were then treated with 1/6 low tox M rabbit complement (Cedarlane Laboratories) for 60 min at 37°C. These cell preparations were spun over Lympholyte M separation medium (Cedarlane Laboratories) to eliminate dead cells. These cell suspensions contained an average of 68% S-Ig positive and 2% Thy 1.2 positive cells.

*Ig assay* – Concentrations of IgM, IgG and IgA in culture supernatants were quantified by a sandwich type ELISA using class specific polyclonal goat anti-mouse Ig antibodies in solid phase (microtiter plates) and the same alkaline phosphatase conjugated antibodies (Zymed Laboratories, San Francisco, CA) as third layer. After addition of p-nitrophenyl phosphate (PPNP, Sigma, Saint-Louis, MI) the optical densities at 405 nm were measured on a Kontron SLT 210 reader. Calibration curves were made with serial dilutions of a pool of normal mouse serum, containing known amounts of IgM, IgG and IgA.

*IL-1 production and assay* – BALB/c spleen cells (from 5 to 10 x 10<sup>6</sup>/ml) were left for 2h in flat bottom 24 well plates at 37°C in a 7% CO<sub>2</sub> humidified atmosphere. The culture medium was made of RPMI 1640 supplemented with 20% heat-inactivated FCS (Seromed GmbH Munich, FRG). Non adherent cells were removed by four successive washings with RPMI 1640. Adherent cells were incubated with LPS, 0111B4, Difco, Detroit, MI or with D53 in CM containing 5% FCS. After 24 to 48h at 37°C, cell-free supernatants were collected and assessed immediately or stored at -70°C.

The assay of IL-1 activity was performed according to Mizel & Mizel (1981). One million thymocytes from 3-6 week old C3H/HeJ mice were cultured in flat bottom microplate wells in CM with Con A (0.5 µg/ml) and the supernatant to be tested at a final dilution of 1:5. Thymocyte proliferation was assessed by measuring <sup>3</sup>H-Thymidine uptake during the last 12h of a 72h culture period at 37°C in a 7% CO<sub>2</sub> humidified atmosphere. Results were expressed as arithmetical mean ± standard deviation of quadruplicate assays.

## RESULTS

**Induction of Il-1 activity by D53** – In a first series of experiments, C3H/HeJ thymocytes were cultured with various concentrations of Con A (from 0.1 to 5  $\mu\text{g}/\text{ml}$ ) with or without D53 at 100  $\mu\text{g}/\text{ml}$ . Results showed that D53 was not mitogenic for thymocytes in the absence of Con A. Furthermore D53 did not act as co-mitogen for thymocytes because it did not potentiate the proliferative response to low doses of Con A (0.1 and 0.5  $\mu\text{g}/\text{ml}$ ) indicating that it did not contain Il-1-like activity. Finally addition of D53 did not decrease thymocyte response to mitogenic concentrations of Con A (1 and 5  $\mu\text{g}/\text{ml}$ ). In a second series of experiments the capacity of D53 to induce adherent spleen cells to produce Il-1 was assessed. Supernatants from adherent cells exposed to D53 (100  $\mu\text{g}/\text{ml}$ ) induced a strong mitogenic response of thymocytes in the presence of Con A (0.5  $\mu\text{g}/\text{ml}$ ). The level of stimulation ( $32,000 \pm 800$  cpm versus  $2900 \pm 200$  cpm in controls) was similar to that achieved with *E. coli* LPS (100  $\mu\text{g}/\text{ml}$ ). The same effect was achieved with *K.p.MPG* with a maximum at 100  $\mu\text{g}/\text{ml}$  and lower but still significant Il-1 stimulation at 10 and 1  $\mu\text{g}/\text{ml}$ . The four ribosomal preparations were tested in this assay at a final concentration of 100  $\mu\text{g}/\text{ml}$ . Each of them but *K.p.* induced a significant Il-1 activity, although to a lesser extent than did *E. coli* LPS or *K.p. MPG* at the same concentrations.

**Spleen cell proliferation** – D53 induced a strong incorporation of  $^3\text{H}$ -Thymidine after 3 days of culture of mouse BALB/c spleen cells (Fig. 1). Maximal stimulation was achieved with D53 at 100 and 500  $\mu\text{g}/\text{ml}$ . At these D53 concentrations  $^3\text{H}$ -Thymidine incorporation was stronger than with *E. coli* LPS at 100  $\mu\text{g}/\text{ml}$ . C3H/HeJ mice which are genetically low responders to *E. coli* LPS responded quite well to D53 (Fig.1). Kinetic studies showed that  $^3\text{H}$ -Thymidine incorporation after stimulation with D53 was similar in C3H/HeJ and in C3H/HeN spleen cells. By contrast the former strain showed a very weak response to *E. coli* LPS (data not shown). Spleen cells from nude mice ( $\text{nu}^+/\text{nu}^+$ ) were strongly stimulated by D53 and to a lesser extent by *E. coli* LPS. As expected such cell suspensions did not respond to PHA. Unlike Con A, neither D53 nor *E. coli* LPS induced significant proliferation of BALB/c thymocytes.

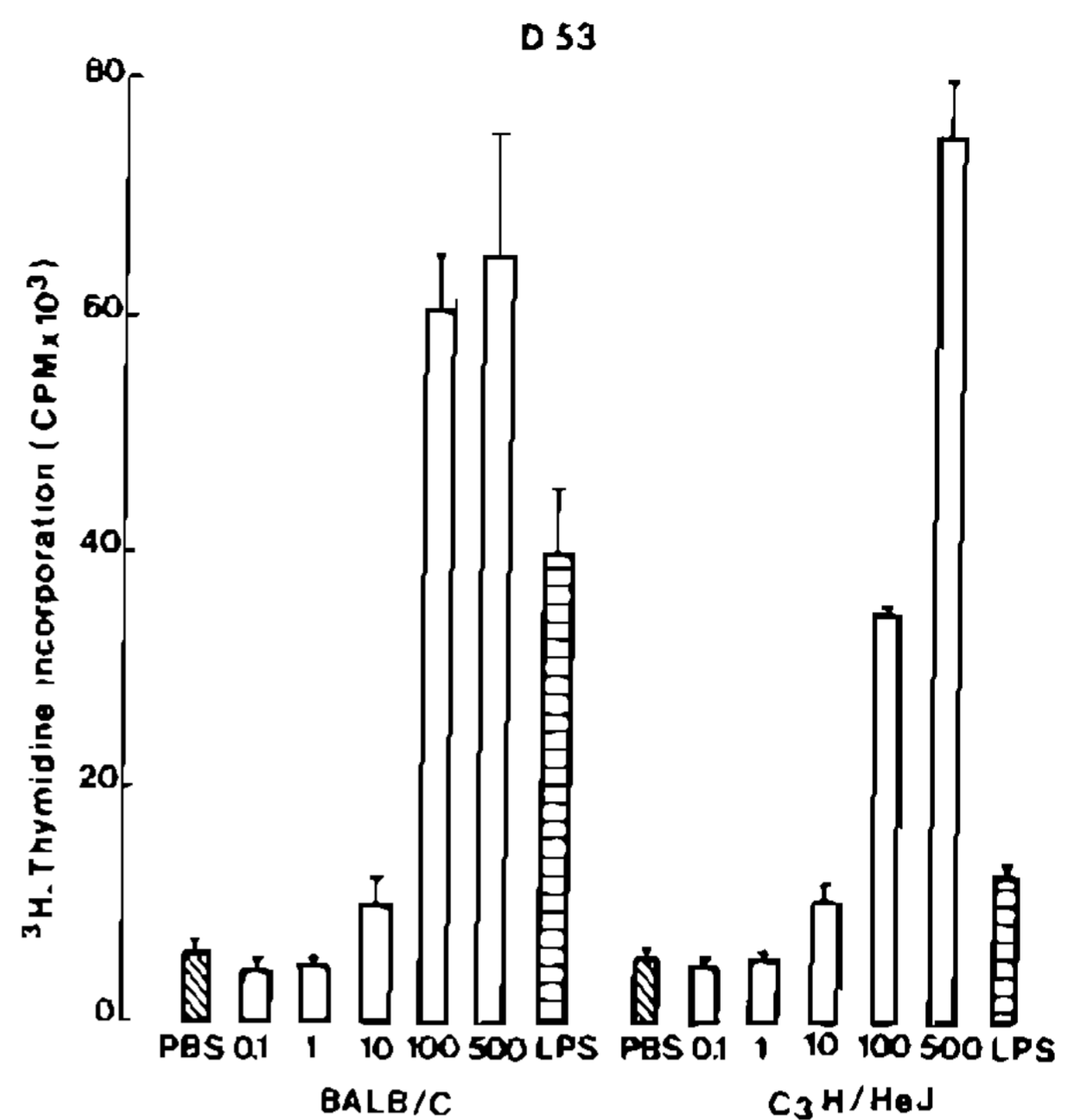


Fig. 1: Mitogenic effect of D53 on BALB/c (left) and C3H/HeJ (right) spleen cells.  $^3\text{H}$ -Thymidine uptake was measured at day 3. Results are expressed as arithmetical means of  $\text{cpm} \times 10^{-3} \pm$  standard error.

In order to know which of D53 components was responsible for the mitogenic activity, we performed BALB/c spleen cell cultures to which *K.p.MPG* or each ribosomal preparation was added at a final concentration of 100  $\mu\text{g}/\text{ml}$ .  $^3\text{H}$ -thymidine uptake was assessed every 24h from day 2 to day 7 of culture. *K.p.MPG* induced the strongest mitogenic response, peaking at day 3 and slowly decreasing thereafter but still quite important at day 7 (Fig. 2). *K.p.* ribosomes induced a mitogenic response of the same order of magnitude as that of LPS, with kinetics similar to that of *K.p.MPG*. Ribosomes from *H.i.* and *St. pyo.* induced a  $^3\text{H}$ -Thymidine uptake of intermediate intensity with different kinetics, peaking at day 2 and then decreasing down to a plateau from day 4-5 to day 7. Finally ribosomes from *St. pn.* induced a low proliferative response with the same kinetics as that of other ribosomes (Fig. 2).

**Fractionation of spleen cell suspensions** – The following experiments were performed to investigate the relative contribution of T and B lymphocytes in the mitogenic effect of D53, as well as the requirement for adherent accessory cells in this effect. The three components of D53 which displayed the strongest mitogenic effects were tested. Removal of most adherent cells yielded suspensions containing less than 1% of macrophages as defined by their morphology after May Grunwald Giemsa staining. Such suspensions responded better than unseparated splenocytes to *K.p.MPG* and equally well to the ribosomes of *K.p.* and *H.i.* (Fig. 3).

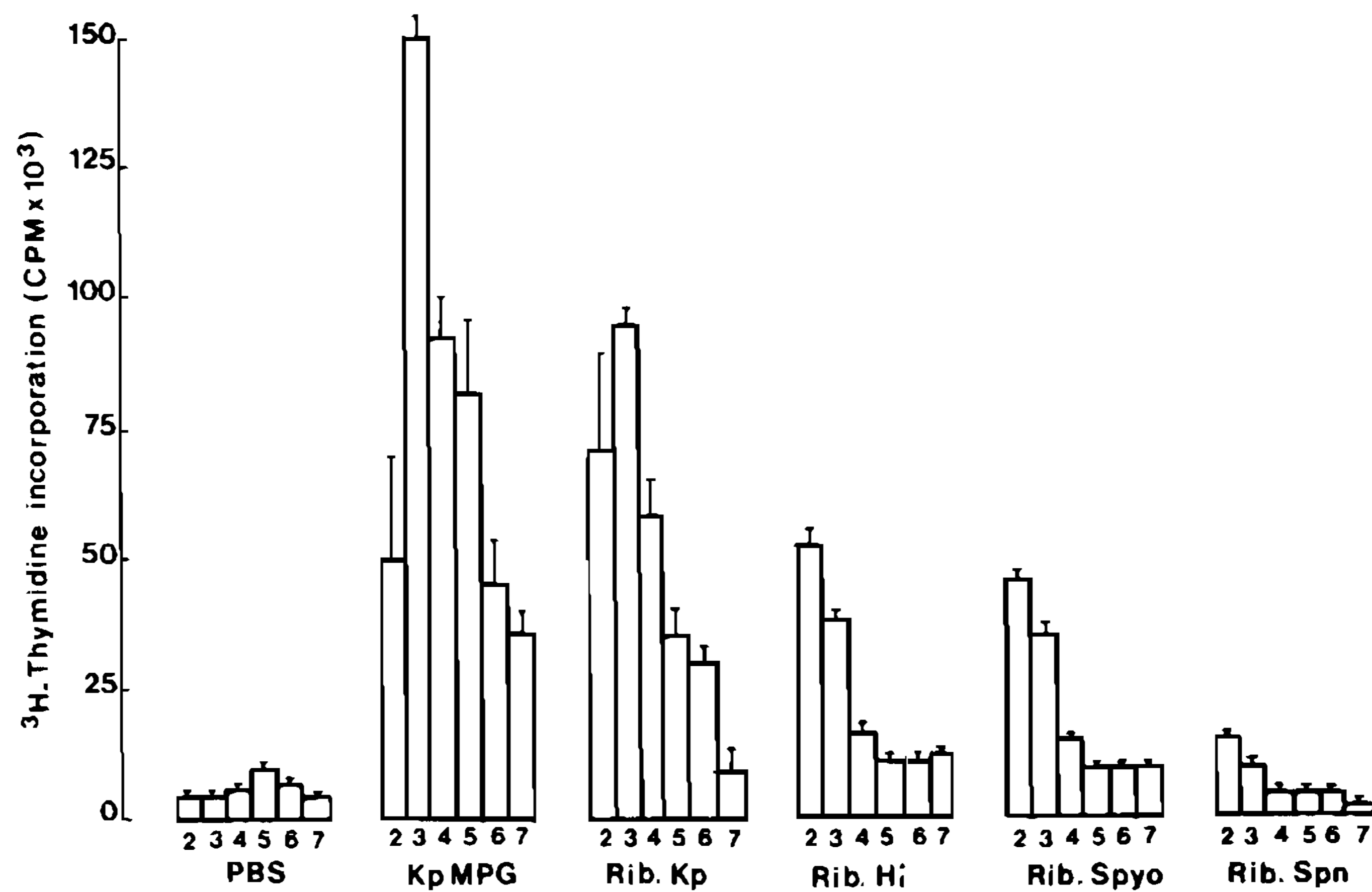


Fig. 2: Mitogenic effect of *K.p.* MPG and ribosomal fractions (Rib) on BALB/c spleen cells. Kinetics of the responses. Each mitogen was at a final concentration of 100  $\mu\text{g/ml}$ . <sup>3</sup>H-thymidine uptake was measured every 24h from day 2 to day 7. Results are expressed as arithmetical means of  $\text{cpm} \times 10^{-3} \pm$  standard error.

Depletion of most B cells by adherence to nylon wool resulted in the nearly complete abrogation of <sup>3</sup>H-Thymidine uptake whereas removal of T cells did not alter the proliferative response (Fig. 3). It was concluded that the bulk of the proliferative response to *K.p.* MPG, *K.p.* and *H.i.* ribosomes could be attributed to B cells. However a possible cooperating effect of T lymphocytes and adherent cells cannot be ruled out by these experiments because the fractionation procedure did not yield pure B cell suspensions. In further experiments positively selected B cells obtained by the "panning" method were cultured with *K.p.* MPG and ribosomes from *K.p.*, *H.i.* and *St.pyo.* strongly stimulated <sup>3</sup>H-Thymidine incorporation. The same results were obtained with Thy 1.2<sup>+</sup> cell depleted suspensions and with unfrac-

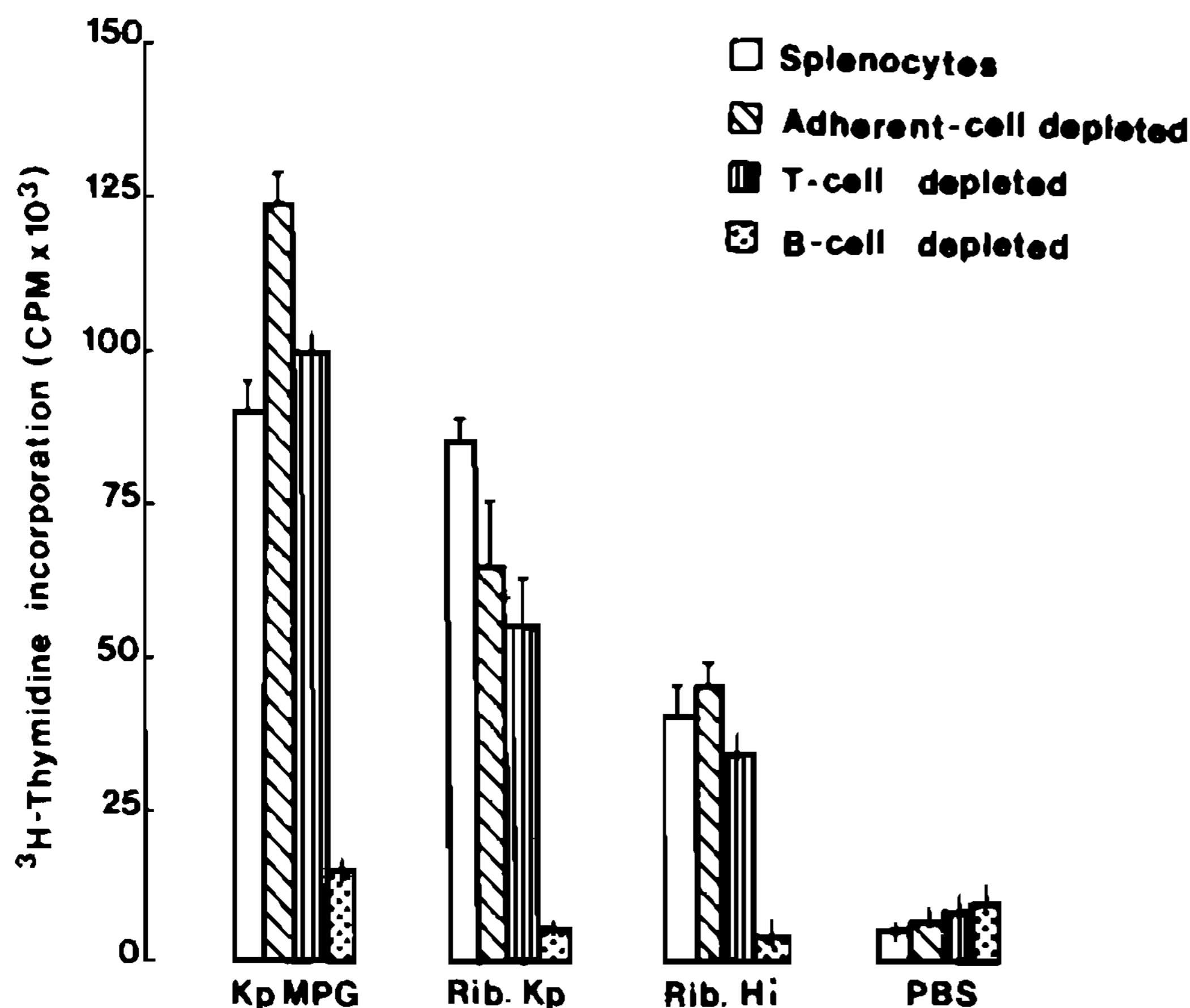


Fig. 3: Mitogenic effect of *K.p.* MPG and ribosomes (Rib) of *K.p.* and *H.i.* assessed on spleen cell suspensions depleted from adherent cells, B cells or T cells.

tionated spleen cells from nude mice. These data indicated that D53 and its components can be regarded as T-independent B cell mitogens.

*Induction of immunoglobulin secretion by D53 and its components* – Supernatants of 5-day cultures of BALB/c spleen cells stimulated by D53 (50  $\mu\text{g/ml}$ ) contained high amounts of IgM. The levels of IgG and IgA were less elevated but still quite above those of unstimulated controls (Table). *K.p.* MPG and ribosomal fractions from *K.p.*, *H.i.* and *St.pyo.* induced Ig secretion. IgM was the predominant class in all cases. Ribosomal fractions induced IgA but no IgM production, whereas *K.p.* MPG stimulation induced more IgG than IgA synthesis (Ta-

TABLE

Immunoglobulin production by BALB/c spleen cells stimulated by D53 components, or *E. coli* LPS

	IgM*	IgG*	IgA*
Control	724	275	1021
<i>K.p.</i> MPG 10 $\mu\text{g/ml}$	21655	12365	2409
<i>E. coli</i> LPS 10 $\mu\text{g/ml}$	11880	1000	1998
Control	448	402	1345
Ribosomes <i>K.p.</i> 100 $\mu\text{g/ml}$	2444	490	1942
Ribosomes <i>H.i.</i> 100 $\mu\text{g/ml}$	3434	652	2137
Ribosomes <i>St.pyo.</i> 100 $\mu\text{g/ml}$	1390	379	1622
Ribosomes <i>St.pn.</i> 100 $\mu\text{g/ml}$	488	219	820

\*Sandwich type ELISA performed with polyclonal heavy chain specific antibodies. Determination on day 5 supernatants. Arithmetical mean (ng/ml) from 2 experiments.

ble). The four fractions induced only IgM secretion by nude spleen cells. Finally ribosomes from *K.p.*, *H.i.* and *St. pyo.* triggered only IgM but no IgG or IgA secretion by spleen cells from CBA/N, a strain which is deficient in the  $lyb5^+$  B cell subset. Ribosomes from *St. pn.* did not trigger any Ig secretion.

#### DISCUSSION

In the present report we show that D53 (Ribomunyl<sup>R</sup>) is a potent inducer of Il-1 production by adherent mouse spleen cells. D53 itself has no Il-1-like activity and does not interfere with the C3H/HeJ thymocyte proliferation assay for Il-1. This property may represent an important part of D53 immunostimulating properties. Indeed Il-1 is one of the major mediator of cellular interactions in the immune system (Oppenheim et al., 1986). Il-1 can be produced by several cell types, especially by monocytes and macrophages upon stimulation by various signals such as lymphokines or bacterial endotoxins. Il-1 exerts multiple biological activities and plays a major role in the initiation of the specific immune response by providing a progression signal in B and T cell activation.

D53 and each of its five components are mitogenic for mouse spleen cells. This activity can be attributed to *K.p.*MPG and to the ribosomes of *K.p.* and *H.i.*, whereas the ribosomes of *St. pyo.* and *St. pn.* induce a weak but significant mitogenic response. The proliferative response cannot be attributed to a secondary in vitro response to antigens to which the animals had been previously exposed because no antibody to D53 or to any of its components could be detected in the sera of experimental mice by using a highly sensitive ELISA technique (data not shown). Most of the proliferating cells are B cells. Moreover D53 does not activate C3H/HeJ nor BALB/c thymus cells whereas nude spleen cells and T-depleted BALB/c spleen cells respond quite well. Of note removal of most macrophages from spleen cell suspensions did not reduce the proliferation induced by *K.p.*MPG and by ribosomes from *K.p.* and *H.i.*

Finally D53 induced the secretion of IgM and to a lesser extent of IgG and IgA by mouse spleen cells. This property can be attributed to *K.p.*MPG and to ribosomal fractions from *H.i.* A low IgM secretion was also induced by ribosomal fractions from *K.p.* and *St. pyo.*, but not by those of *St. pn.* Since D53 induced B cell proliferation and Ig secretion in non-immunized

animals, it can be regarded as a polyclonal B cell activator.

The capacity of D53 to induce Il-1 production and polyclonal B cell activation is comparable to that of *E. coli* LPS which represents the reference positive control in the assays (McKearn et al., 1982). However these properties cannot be accounted for by LPS contamination of D53 because (i) the magnitude of the response to D53 was at least equal to that of LPS at the same concentration and (ii) the C3H/HeJ mice which bear a defective *lps* gene and show a low response to *E. coli* LPS were strongly responsive to D53. Therefore the mitogenic components of D53 and LPS should be regarded as two distinct polyclonal B cell activators.

#### ACKNOWLEDGEMENTS

The technical assistance of M. Jeannin and A. de Fraissinette is acknowledged. Part of the data including Figs. 1, 2 and 3 are reproduced from "Recent advances in immunotherapy: bacterial ribosomal fractions" John Libbey Eurotext Ltd. 1987 3 :37-43.

#### REFERENCES

- BOUSQUET, J. & DUSSOURD d'HINTERLAND, L., 1982. Prévention de l'infection bactérienne chronique des voies respiratoires par un vaccin ribosomal. *Med. Hyg.*, 40 :146-157.
- DUSSOURD d'HINTERLAND, L.; NORMIER, G. & DURAND, J., 1980. Ribosomal vaccines: preparation of sub-cellular fractions. *Arzneim. Fors.* 30 :126-132.
- DUSSOURD d'HINTERLAND, L.; NORMIER, G.; PINEL, A.M. & WIGZELL, H., 1985. NK cell stimulant properties of a membrane proteoglycan from non capsulated *Klebsiella pneumoniae* biotype a. *Acta Pathol. Microbiol. Immunol. Scand. Section C*, 93 :233-243.
- JULIUS, M.H.E.; SIMPSON, E. & HERZENBERG, L. A., 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.*, 3 :645-649.
- Mc KEARN, J.P.; PASLAY, J.W.; SLACK, J.; BAUM, C. & DAVIE, J.M., 1982. B cell subsets and differential responses to mitogens. *Immunol. Rev.*, 64 :5-23.
- MICHEL, F.B.; DUSSOURD d'HINTERLAND, L.; BOUSQUET, J.; PINEL, A.M. & NORMIER, G., 1978. Immunostimulation by a ribosomal vaccine associated with a bacterial cell wall adjuvant in humans. *Infect. Immunol.*, 20 :760-769.
- MIZEL, S.B. & MIZEL, D., 1981. Purification to apparent homogeneity of murine interleukin-1. *J. Immunol.*, 126 :834-837.
- OPPENHEIM, J.J.; KOVACS, E.J.; MATSUSHIMA, K. & DURUM, S.K., 1986. There is more than one interleukin-1. *Immunol. Today*, 7 :45-56.
- PINEL, A.M.; NORMIER, G.; DUSSOURD d'HINTERLAND, L.; LINDQVIST, C. & WIGZELL, H.,

- 1985a. Hybridoma antibodies against protective and non protective antigenic determinants of *Streptococcus pyogenes* ribosomal vaccine. *J. Int. Soc. Immunopharmacol.*, 7 :400.
- PINEL, A.M.; NORMIER, G.; DUSSOURD d'HINTERLAND, L.; LINDQVIST, C. & WIGZELL, H., 1985b. Hybridoma antibodies against protective antigenic determinants of a *Klebsiella pneumoniae* ribosomal vaccine. *J. Int. Soc. Immunopharmacol.*, 7 :400.
- WYSOCKI, L.J. & SATO, V.L., 1978. "Panning" for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. USA*, 75 :2844.